IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re: Patent Application

Applicant(s):	Maino et al.	Docket No.:	P-3639P1
Serial No.:	08/803,702	Group Art Unit:	1644
Filing Date:	February 21, 1997	Examiner:	Gerald Ewoldt, Ph.D.
For:	METHOD FOR DETECTING T CELL RESPONSE TO SPECIFIC ANTIGENS IN WHOLE BLOOD		

TRANSMITTAL of CORRECTED APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Submitted herewith are the following enclosure(s):

- **Appeal Brief (49 numbered pages & 6 References)**

Total number of pages: **114**

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Jolanta Pence
(Print Name)

Jolanta Pence
(Signature)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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APPEAL BRIEF UNDER 37 C.F.R. §41.37

Sir:

Appellants submit this brief in support of their notice of appeal dated July 30, 2004, pursuant to 37 C.F.R. §41.37.

In view of the arguments and authorities set forth below, this Board should find the Final Rejections of the appealed claims, as specified below, to be in error and should reverse them.

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(i) Real party in interest

The real party in interest consists of Becton, Dickinson and Company, which owns an undivided partial interest in the present application by virtue of an assignment from co-inventors Maino and Suni, and the Board of Regents, University of Texas System, which holds an undivided partial interest in the present application by virtue of an assignment from co-inventor Picker.

An assignment from co-inventors Maino and Suni to Becton, Dickinson and Company was recorded June 9, 1997 at Reel 8546, Frame 0073. An assignment from co-inventors Maino and Suni to Becton, Dickinson and Company also was recorded May 8, 2000 at Reel 010790, Frame 0936.

The assignment from co-inventor Picker to the Board of Regents, University of Texas System, was not recorded.

(ii) Related appeals and interferences

There are no prior or pending appeals, interferences or judicial proceedings known to Appellants, Appellants legal representative, or Assignee which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

(iii) Status of claims

The status of all the claims, pending or cancelled, is as follows:

Claims 19-24, 26-33, 35-55, and 61-66 are pending.

Claims 1-18, 25, 34, and 56-60 are canceled.

Claims 19-24, 26-33, 35-55, and 61-65 stand rejected.

Claim 66 is allowed.

The rejections of Claims 19-24, 26-33, 35-55, and 61-65 are being appealed.

(iv) Status of amendments

Appellant's amendment under 37 C.F.R. §41.33(b)(2) submitted on September 22, 2004 was entered. In response, an advisory action was issued October 14, 2002, stating that claim 66 now is allowed.

(v) Summary of claimed subject matter

Appellants' invention is in the field of immunology and provides methods for identifying and quantifying T lymphocytes (equivalently "T cells") that are specific for a particular antigen of interest, such as from an infectious agent (e.g., a virus) or administered in a vaccine. To aid in understanding the claimed invention, Appellants' first provide, as background, a summary of terms and concepts from the field of immunology relevant to the claimed invention. Appellants then provide a concise explanation of the subject matter defined in each of the independent claims involved in the appeal, which are claims 19, 64, and 65.

Background

T cells (equivalently "T lymphocytes") are groups of blood cells that are involved in the immune system's antigen-specific response to foreign (i.e., non-self) antigens. T cells are among the subclass of blood cells referred to as peripheral blood mononuclear cells (PBMC's).

Each T cell recognizes a specific antigen (its "cognate antigen") through a receptor on its surface, termed the "T cell receptor" ("TCR"), capable of binding to the antigen. Although T cells collectively recognize a phenomenal diversity of foreign antigens, each individual T cell recognizes a single discrete antigen, the specificity of recognition resulting from the specificity of the TCR-antigen binding. A T cell that recognizes a particular antigen is said to be specific for the antigen. The present invention relates to detecting those small number of T cells that recognize a specific antigen out of the totality of T cells present in a sample of blood (or PBMC-containing fraction of the blood).

T cell antigen binding is mediated by antigen presenting cells (APC's), also among the PBMC's, which process protein antigens into peptide fragments, complex the peptide fragments with major histocompatibility ("MHC") molecules, and display the resulting complexes on the cell surface. TCR's only bind peptide fragments of proteins that are complexed with MHC molecules and displayed by an APC. Antigens that are processed within an APC and displayed on the surface complexed with MHC molecules

are referred to as "nominal antigens" and are the antigens that evoke a classical antigen-specific immune response by T cells. The present invention, more particularly, relates to detecting those small number of T cells that recognize a specific nominal antigen out of the totality of T cells present in a sample of blood.

Binding of a T cell to its cognate antigen complexed with MHC molecules on the surface of an APC leads to activation of the T cell. Activation triggers biochemical and morphological changes in the T cell, including changes in protein expression, that culminate in differentiation of T cells into one of various types of effector cells.

Two classes of molecules whose expression are changed during activation are (1) cytokines, which are signaling proteins secreted from the T cell that mediate the immune response, and (2) proteins that appear on the surface of the activated T cell surface, referred to as activation antigens.[1] Detection of activation-induced protein expression facilitates identification of activated T cells. However, the two classes of molecules do not identify the same T cell population. In particular, CD69, an early activation antigen, is expressed within hours of T cell activation. However, only a subset of the CD69-expressing cells go on to secrete cytokines and fully mature into effector cells (see, e.g., the specification at page 8, lines 12-21). The methods of the present invention relate to the detection of the antigen-specific T cells that produce certain cytokines in response to stimulation with the nominal antigen.

In addition to presenting a nominal antigen bound to MHC molecules for recognition by a T cell, an APC also presents cell-surface molecules that contribute to the activation of the T cell. These cell surface molecules are said to provide costimulation. The addition of additional costimulatory molecules into the incubation buffer to further augment T cell activation is referred to exogenous costimulation.

T cells can be activated in other ways than with a nominal antigen presented by an APC. One class of activating agents, referred to polyclonal activators (equivalently, "mitogens"), bypass the T cell receptor and activate large fractions of T cells regardless of the T cells' antigen specificity. Another class of activating agents, termed

[1] The nomenclature is somewhat confusing: an activation antigen, which is a protein expressed on the surface of an activated T cell, should not be confused with a nominal antigen, which is a protein recognized by a T cell in a process that leads to activation.

superantigens, activate large subsets of T cells without regard to the T cell's antigen specificity. Thus, the subset of T cells identified by detecting activation-induced protein expression depends on the class of agent that evoked activation. In the methods of the present invention, T cells specific for a nominal antigen are detected as those T cells that produce certain cytokines in response to stimulation with the nominal antigen.

The methods of the present invention are distinguished in part from prior art methods by both the class of agent used to evoke activation and the subset of T cells detected. The closest prior art of record describes methods for detecting T cells activated in a non-specific manner by detecting the expression of intracellular cytokines following stimulation by contact with a polyclonal activator or superantigen, which activates a vastly larger subset of the T cells in a sample. In these prior art methods, an inhibitor of cytokine secretion was used to allow intracellular cytokines to accumulate in the T cells, thereby facilitating the detection of the intracellular cytokines.

In the present invention, the frequencies of antigen-specific T cells are assessed flow cytometrically. Flow cytometry is a well known method of analyzing cells essentially one at a time that involves passing the cells in a fluid stream past a detection region and measuring various features of each cell as it passes. Prior to analysis, particular cellular components, such as intracellular or cell-surface proteins, are made visible to the detectors by labeling, typically with fluorophores (fluorescent dyes). The labeling of a particular protein in or on a cell is carried out using an antibody that binds specifically to this protein; typically the antibody is conjugated, directly or indirectly, to a fluorophore. Thus, the detection of a particular protein is achieved by adding a protein-specific antibody to the sample and detecting the binding of the antibody to the protein, which binding indicates the presence of the protein. Similarly, subsets of cells defined by the expression of a particular protein can be identified by adding an antibody specific to the subset-defining protein (also referred to as a subset-defining antibody) and detecting the binding of the antibody to the protein.

Using multiple antibodies specific to different proteins, each specificity labeled with a measurably distinct fluorophore, multiple proteins in a cell can be detected simultaneously. During a flow cytometric analysis, as a cell, which may have one or more of its proteins labeled, passes through the detection region, the cell is exposed to an

excitation light that causes any fluorophore labels, if present, to fluoresce (emit light), and the resulting fluorescent emission is measured as an indication of the presence of labeled protein. The amount of fluorescence, i.e., the intensity, observed from a fluorophore used to label a cellular protein corresponds to the amount of that protein in or on the cell. In addition, excitation light scattered by the cell itself typically is measured and provides additional information characteristic of the cell type.

Flow cytometric analysis yields data in the form of a set of emission intensity values measured for each cell, both from light scattered by the cell and from fluorescence emissions from the various labels. The detection of a cell by the detector also is referred to as an event, and measuring the cell parameters also is referred to as measuring events. Although a number of cellular parameters can be measured simultaneously, the resulting data typically are displayed two parameters at a time in a dot-plot in which each cell (event) is represented as a point with coordinates of the point corresponding to two intensity values. Examples of such dot-plots are provided in Figures 1-3 or the specification. Cell frequency within a subset is assessed by counting the number of cells of the desired type, each identified as having measured emission values characteristic of the subset, and comparing that the total number of cells observed.

Subject Matter of the Independent Claims

Of the pending claims, claims 19 and 64-66 are independent. Claim 66 is allowed and is not under appeal.

Claim 19

Independent claim 19 is drawn to a method of detecting T lymphocytes ("T cells") that are specific for a nominal antigen. The method of the present invention is described in broad terms in the specification at page 4, lines 10-22, and in originally filed claim 1. Claim 19 further recites additional steps, as described below. Claim 19, with steps numbered for reference, is shown below:

Claim 19. A method of detecting T lymphocytes that are specific for a nominal antigen, comprising:

1. culturing a sample containing peripheral blood mononuclear cells with a nominal antigen;
2. adding to said sample an inhibitor of cytokine secretion;
3. permeabilizing said cells;
4. adding to said sample at least one cytokine-specific antibody and at least one T lymphocyte subset-defining antibody; and then
5. flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset.

In step 1 of claim 19, a sample containing peripheral blood mononuclear cells ("PBMC") and, thus, including both lymphocytes and antigen-presenting cells, is cultured with a nominal antigen. T cells that are specific for the stimulating nominal antigen are activated and begin to synthesize cytokines.

In step 2 of claim 19, an inhibitor of cytokine secretion is added, which results in the intracellular cytokines expressed within the activated T cells to accumulate, thereby enhancing detection. This step is described in the specification at page 5, lines 15-17.

In step 3 of claim 19, cells are permeabilized to allow antibodies added to the sample (in the following step) to enter the interior of the cell, thus enabling antibodies specific for intracellular proteins to bind to their target proteins, if present. This step is described in the specification as part of the preferred methodology at page 7, step 4.

In step 4 of claim 19, at least one cytokine-specific antibody and at least one T cell subset-defining antibody are added to the sample. This step is described as part of the general method at page 4, lines 10-15, and as part of the preferred methodology at page 7, step 6. T cell subset-defining antibodies, which bind to T cells independently of their activation status, may be chosen to bind, for example, to a protein expressed on all T cells, such as the CD3, or may, in addition or in the alternative, be chosen to bind to a protein is expressed on a smaller subset of T cells, such as CD4 or CD8, as described in the specification at page 3, lines 7-15; page 8, lines 15-18; and page 12, lines 10-20.

Examples of cytokine-specific antibodies are antibodies specific for IL 2, IL-4, IL-13, γ-IFN, or TNF-α, as disclosed in the specification at page 3, lines 7-15.

In step 5 of claim 19, flow cytometry is used to detect simultaneously the intracellular binding of the cytokine-specific antibody and the binding of the subset-defining antibody. Cells to which both types of antibodies bind are identified as T cells (or subset thereof) and as activated. The purpose of the method, the detection of T cells specific for the nominal antigen, is achieved because only T cells that are activated by the nominal antigen express the intracellular cytokines that are detected through the binding of the cytokine-specific antibodies. The detection step is described in the specification as part of the general method at page 4, lines 10-15, and as part of the preferred methodology at page 7, step 8, and in Example 3.

Claim 64

Claims 64 corresponds to claim 19 further limited to the use of the preferred inhibitor of cytokine secretion, Brefeldin-A, as described in the specification at page 5, lines 17, and exemplified throughout.

Claim 65

Claim 65 corresponds to claim 64 further narrowed to the method in which the step of culturing a sample containing peripheral blood mononuclear cells with a nominal antigen in the presence of Brefeldin-A is carried out in a slant tube, as in the preferred methodology described in the specification at page 6, step 3.

(vi) Grounds of rejection to be reviewed on appeal

Ground 1: Claims 19-24, 26-33, 35-36, 40-55, and 61-63 were rejected under 35 U.S.C. §112, first paragraph, for lack of adequate written description.

Ground II. Claims 19-24, 26-33, 35-55, and 61-65 were rejected under 35 U.S.C. §112, first paragraph, for lack of enablement.

(vii) Argument.

Ground I. The rejection of claims 19-24, 26-33, 35-36, 40-55, and 61-63 under the written description requirement of 35 U.S.C. §112, first paragraph.

Claims 19-24, 26-33, 35-36, 40-55, and 61-63 were rejected under the written description requirement of 35 U.S.C. §112, first paragraph, on the ground that the specification provides insufficient written description for the term "inhibitor of cytokine secretion." It is the examiner's position that this term potentially could encompass a large number of chemical compounds, and suggested that the claimed genus has not been adequately described.

The legal standard

Section 112 of the patent statute sets forth that "The specification shall contain a written description of the invention". 35 U.S.C. §112, first paragraph. This requirement is separate and distinct from the enablement requirement. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991).

An objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989). Under *Vas-Cath Inc. v. Mahurkar*, supra, to satisfy the written description requirement, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed.

It is well established that the description need only describe in detail that which is new or not conventional. *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). This is equally true whether the claimed invention is directed to a product or a process.

Where claims are drawn to novel combinations or uses of known compounds, in contrast to claims drawn to classes of new compounds per se or claims drawn to processes using those new compounds, the specification need not describe in detail the

known compounds themselves. *In re Fuetterer*, 319 F.2d 259, 138 USPQ 217, (CCPA 1963), discussed in further detail, below.

Claims 19-24, 26-33, 35-36, 40-55, and 61-63

Claims 19-24, 26-33, 35-36, 40-55, and 61-63 stand or fall together with respect to the present rejection. Claim 19 is in independent form; claims 20-24, 26-33, 35-36, 40-55, and 61-63 depend directly or indirectly from claim 19.

The specification provides literal written description of the invention using an inhibitor of cytokine secretion, and exemplifies the use of the preferred inhibitor, Brefeldin A ("BFA"). In particular, the invention using an inhibitor of cytokine secretion is described in the specification at page 5, lines 15-17:

> Further, it has been found intracellular cytokine detection is enhanced when an agent which blocks the secretion of such intracellular cytokines is added during the activation (4h) period of incubation.

In addition, written description of the method of the invention using an inhibitor of cytokine secretion is found in original claim 13, as filed.

> Original Claim 13: The method of Claim 1 further comprising adding a reagent which blocks secretion of intracellular cytokines during the culturing of the sample with the antigen specific stimulus.

The literal description of the invention as claimed in claim 19, using an inhibitor of cytokine secretion, provided both in the specification and the claims as filed conveys with reasonable clarity to those skilled in the art that, as of the filing date, the inventors were in possession of the invention as claimed in claim 19.

Despite the written description provided by the specification showing that the inventors were in possession of the claimed invention, the examiner rejected the claims for lack of written description of the class of compounds encompassed with "an inhibitor of cytokine secretion". Appellants' contend that the rejection is erroneous for the following reasons, discussed more fully below:

1. The use of an inhibitor of cytokine secretion to allow intracellular cytokines to accumulate, thereby facilitating the detection of the intracellular cytokines, was known at the time of the invention. The description need only describe in detail

that which is new or not conventional (*Hybritech v. Monoclonal Antibodies*, supra).

2. The claims, read as a whole, are drawn to a new use of known compounds (an inhibitor of cytokine secretion) and are *not* drawn to either novel compounds per se or to methods using novel compounds. In such a case, the applicant is not required to discover all the compounds from this class that would be useable in the methods (*In re Fuetterer*, supra).

The use of an "inhibitor of cytokine secretion" to facilitate detection of intracellular cytokines was known in the art

The invention of independent claim 19 is a method involving the detection of T cells activated in an antigen-specific manner by detecting the expression of intracellular cytokines following stimulation by contact with a nominal antigen. In the claimed method, an inhibitor of cytokine secretion is used to allow intracellular cytokines to accumulate, thereby facilitating the detection of the intracellular cytokines.

The closest prior art of record describes methods involving the detection of T cells activated in a non-specific manner by detecting the expression of intracellular cytokines following stimulation by contact with a polyclonal activator or superantigen. In these prior art methods, an inhibitor of cytokine secretion was used to allow intracellular cytokines to accumulate, thereby facilitating the detection of the intracellular cytokines. References of record that describe such methods, along with the particular inhibitor of cytokine secretion used, are provided in the table, below.

Inhibitor used	**Reference**
Monensin	Jung et al., 1993, J. Immunol. Methods 159:197-207
Monensin	Elson et al., 1995, J. Immunol. 154(9):4294-4301
Monensin	Prussin et al., 1995, J. Immunol. Methods 188:117-128
Brefeldin-A	Picker et al., 1995, Blood 86:1408-1419
Brefeldin-A & monensin	Application Note 1: Detection of Intracellular Cytokines in Activated Lymphocytes, Becton Dickinson and Co.

Each of the references listed in the table, above, describe methods involving the detection of intracellular cytokines in which an inhibitor of cytokine secretion is used to

allow intracellular cytokines to accumulate in order to facilitate detection. Although the claimed methods are distinguished from these earlier methods both in the specificity of activation and the subset of T cells detected, the claimed methods include the use of an inhibitor of cytokine secretion for the same purpose as previously described, to allow intracellular cytokines to accumulate in T cells in order to facilitate detection. This particular element of the present invention is old in the art and conventional to one of skilled in the art of detecting intracellular cytokines[2].

The rejection is in error because it is based on an improper requirement to describe in great detail an element of the invention which is old in the art. Furthermore, the rejection conflicts with the precedent set by the Court in *Hybritech v. Monoclonal Antibodies*, supra, "The description need only describe in detail that which is new or not conventional". For at least this reason, Appellants submit that the rejection is erroneous and should be overturned.

The claims are drawn to a new use of known compounds

Independent claim 19 is drawn to a novel method that includes the known use of an inhibitor of cytokine secretion, not to the discovery of compounds useful as inhibitors of cytokine secretion. The examiner acknowledged that the present invention is a new use of old elements:

> In fact, none of the individual pieces of the claimed method are actually new [...] What is new is the *combination* of techniques and steps that achieve an unexpected result, i.e., the detection of cytokines that were thought to exist at levels below the threshold of detectability.

Last Office action, page 3, first paragraph. Thus, the claims are analogous to those at issue in *In re Fuetterer*, supra, wherein the Court clarified the written description requirement with respect to claims drawn to novel combinations or uses of known compounds, in contrast to claims drawn to classes of new compounds per se or claims drawn to processes using those new compounds.

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[2] Appellants further note that the fact that this element is old in the art is not in dispute. The examiner stated, "In fact, none of the individual pieces of the claimed method are actually new [...] What is new is the *combination* of techniques and steps that achieve an unexpected result, i.e., the detection of cytokines that were thought to exist at levels below the threshold of detectability." Last Office action, page 3, first paragraph.

In *In re Fuetterer*, claims drawn to a rubber stock composition useful in producing tire treads included a recitation of "an inorganic salt capable" of maintaining an homogeneous distribution of another component in the composition. The disclosure listed the function desired and four members of the class having that function. The claims had been rejected by the examiner as being overly broad ("'inorganic salt" reads on literally thousands of materials, many of which would not be operative for applicant's purpose'. Ibid at 220). The board agreed, noting that rejection was based on "the inordinate breadth of the claimed salts when it is not apparent from the disclosure of only four salts what other salts would be suitable to serve the function asserted and required by the claims" (Ibid at 220, 221). However, the Court overturned the rejection and found the written description requirement to be satisfied:

> Appellant's invention is the combination claimed and not the discovery that certain inorganic salts have colloid suspending properties. We see nothing in patent law which requires appellant to discover which of all those salts have such properties and which will function properly in his combination. The invention description clearly indicates that any inorganic salt which has such properties is usable in his combination. If others in the future discover what inorganic salts additional to those enumerated do have such properties, it is clear appellant will have no control over them per se, and equally clear his claims should not be so restricted that they can be avoided merely by using some inorganic salt not named by appellant in his disclosure.

Ibid at 223 (USPQ pagination) (emphasis added). Appellants submit that the facts in the present case are analogous to those in *In re Fuetterer*.

As in *In re Fuetterer*, the present claims stand rejected in view of the large number of potential inhibitors of cytokine secretion. Analogously, the present claims are to a combination of steps, one being the known use of an inhibitor of cytokine secretion, not to the discovery of compounds that act as inhibitors of cytokine secretion. In accord with in *In re Fuetterer*, nothing in patent law requires Appellants to discover which of all those potential inhibitors of cytokine secretion have such properties and which will function properly in the claimed combination of steps. Furthermore, if others in the future discover another suitable inhibitor of cytokine secretion, the present claims should not be so restricted that they can be avoided merely by using some inhibitor of cytokine secretion not described in the specification. The rejection is in error because it is based on an erroneous standard and is at odds with the precedent set by the Court in *In re*

Fuetterer. For at least this reason, Appellants submit that the rejection is erroneous and should be overturned.

Appellants suggest an analogous fact pattern that may clarify the error of the rejection. Consider hypothetical claims drawn to a novel multi-step method of treating a patient in which one step is treating a headache using a pain reliever, supported in the specification by a teaching to use "a pain reliever, such as aspirin." The use of a pain reliever to treat a headache is, of course, well known and several suitable compounds are commercially available; the teaching of the specification would be sufficient to convey to one of skill that this step can be carried out using any of suitable pain relievers known. While it is true that only a very small number of medically suitable compounds are known out of the "infinity" of potential compounds, and, in fact, the full scope of the term "pain reliever" is not known (hence, the continuing research into new compounds), the hypothetical invention simply comprises the use of a known pain-reliever for its known purpose. The teaching in the present specification to use an inhibitor of cytokine secretion, such as BFA, is analogous to a teaching in the hypothetical specification to use a pain reliever, such as aspirin - both teach the use of a known compound to carry out a known step. The present rejection is analogous to an improper requirement that the inventor of this hypothetical invention describe in detail the class of compounds suitable for use as a pain reliever.

In summary, the literal description in the specification reasonably conveys to one of skill in the art that Appellants were in possession of methods using "an inhibitor of cytokine secretion". This element of the claimed invention, specifically the use of an inhibitor of cytokine secretion to achieve an accumulation of intracellular cytokines to facilitate detection, was known at the time of the invention; the specification need not describe this known element in detail. Furthermore, the claims are to a combination of elements, one being the known use of an inhibitor of cytokine secretion, not to the discovery of compounds that act as inhibitors of cytokine secretion; again, the specification need not describe this known element in detail. The rejection is based on an erroneous standard that conflicts with the precedent of the case law cited.

For the reasons discussed herein and in view of the case law cited, Appellants submit that the specification fully meets the written description requirement. Appellants respectfully request that the Board overturn the rejection of claims 19-24, 26-33, 35-36, 40-55, and 61-63 under the written description requirement of 35 U.S.C. §112, first paragraph.

Ground II. The rejection of claims 19-24, 26-33, 35-55, and 61-65 under the enablement requirement of 35 U.S.C. §112, first paragraph.

Claims 19-24, 26-33, 35-55, and 61-65 were rejected under 35 U.S.C. §112, first paragraph, for lack of enablement, based on the grounds that a disclosed critical limitation is missing from the claims. As stated in the Office action dated April 28, 2004 (referred to herein as "the last Office action"):

> Elements critical or essential to the practice of the invention, but not included in the claims are not enabled by the disclosure, for the reasons of record.

Last Office action, page 4, §5.

However, which elements are alleged to be critical is unclear. In restating the "reasons of record", the examiner restated the rejection as set forth in the Office action dated December 2, 2002, and additionally provided comments responding specifically to the arguments set forth in Appellants' amendment dated February 17, 2004. No acknowledgement was made of the prosecution history in the interim, namely Appellants' amendments dated March 31, 2003 and September 3, 2003, and the Office actions dated June 13, 2003 and October 14, 2003. It is Appellants' understanding that the examiner had agreed that particular features alleged to be critical in the Office action dated December 2, 2002 are, in fact, not critical features and, thus, Appellants' believe their mention in the restated reasons for the rejection was unintentional.

Clarity as to the particular elements alleged to be critical is necessary in order for Appellants to respond to the rejection with appropriately grouped claims, as the elements are incorporated into the claims in various combinations. Thus, both for completeness and in order to be able to group claims, Appellants first address all elements mentioned in the restatement of the rejection and summarize Appellants' understanding of the reasons for the rejection.

Appellants' understanding of the reasons for the rejection

The reasons for the rejection restated from the Office action dated December 2, 2002 (last Office action, §5, paragraph spanning pages 4-5) included the following elements as allegedly critical elements omitted from one or more claims:

1. the step of culturing the sample with the antigen;

2. added costimulation ("as recited in Claim 20");

3. the inclusion of an inhibitor of cytokine secretion; and

additional required steps described in Example 4, "for example"

4. the use of slant tubes

5. the inclusion of CD69 (not just any activation marker)

6. the collection of at least 50,000 events

Of these elements, Appellants believe that elements 1, 2, and 5 are not presently alleged to be critical, for the reasons summarized below.

Regarding element 1, the step of culturing the sample with the antigen, claim 19 was amended to recite the step of culturing the sample with the nominal antigen in the amendment dated March 31, 2003. The examiner acknowledged the amendment and explicitly withdrew this as a reason for the rejection in the Office action dated June 13, 2003 (page 4, last paragraph). Appellants understand that this element is not alleged to be an omitted critical element.

Regarding element 2, the step of adding (i.e., exogenous) costimulation, the examiner earlier had stated, without support, that "it is well-established that antigen stimulation in the absence of costimulation (....) will result in anergy (not activation)" (Office action dated December 2, 2002, page 5). In the amendment dated March 31, 2003, Appellants pointed out that costimulation inherently is provided by antigen presenting cells (APC) in the sample, making the addition of an exogenous costimulant (as recited in Claim 20) optional. This element was not cited as a reason for the rejection in the subsequent Office actions dated June 13, 2003 and October 14, 2003, and Appellants understand that this element is no longer alleged to be critical.

Regarding the additional steps described in Example 4, this example describes four key areas in which the methods of the invention, which involve detecting T cell cytokine expression in response to a nominal antigen, differ from the prior art methods that involve detecting T cell cytokine expression in response to polyclonal stimulators: (1) the geometry of the T cell/accessory cell interaction (specification at page 16, lines 13-16); (2) the timing of the addition of Brefeldin-A and the use of exogenous costimulation (specification at page 16, lines 16-18); (3) assessment of CD69 (specification at page 16, lines 18-26); and (4) the number of events collected

(specification at page 16, line 26 to page 17, line 2).[3] Appellants provided arguments showing that the teaching of Example 4, and, in particular, regarding these 4 areas, is not a description of critical elements whose omission would result in the methods being wholly inoperable (restated in the amendment dated February 17, 2004). In responding to Appellants arguments, the examiner explicitly stated that the timing of BFA inclusion and the assessment of CD69 (element 5, above) are not critical features because the specification does not disclose it a such (last Office action, page 6). Appellants understand that the use of exogenous costimulation also is not alleged to be critical, both for the reasons stated above and further because this feature is described in the same sentence in Example 4 together with the timing of BFA inclusion, which description the examiner stated does disclose a critical element.

Thus, it is Appellants understanding that the sole reasons for the rejection are the alleged criticality of

- the use of slant tubes
- the measurement of at least 50,000 events
- inclusion of an inhibitor of cytokine secretion

and that the alleged criticality is based on the description of these features in Example 4.

The legal standard

35 U.S.C. §112, first paragraph, sets forth that the specification, not the claims, must provide an enabling written description of the invention:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

35 U.S.C. §112, first paragraph.

The examiner cited *In re Mayhew*, 527 F.2d 1229, 1233, 188 USPQ 356, 358 (CCPA 1976) as supporting a rejection of claim under the enablement provision section of 35 U.S.C. §112 when a feature which is taught as critical in a specification and is not

[3] The last paragraph of Example 4 (page 17, lines 4-17) provides further guidance directed to a particular narrower embodiment of the invention in which the sample is whole blood. Appellants believe that this is not relevant to the rejection.

recited in the claims. In *In re Mayhew*, the specification contained multiple teachings that the invention was dependent on a specific feature (a cooling zone, specifically located), and this feature was omitted entirely in the broadest claim. The teachings of the specification, considered in its entirety, indicated that the best mode (i.e., with a cooling zone present) was, in fact, the only mode supported.

The applicability of the reasoning of *In re Mayhew* depends on a proper determination of whether a feature actually is a critical feature, i.e., whether the specification teaches that the invention truly depends on the inclusion of the critical feature. A determination that a disclosed limitation is critical should be made only when the language of the specification, taken as a whole, makes it clear that the limitation is critical for the invention to function as intended:

> In determining whether an unclaimed feature is critical, the entire disclosure must be considered. Broad language in the disclosure (including the abstract) omitting an allegedly critical feature tends to rebut the argument of criticality. Also, features that are merely preferred are not critical.

In re Goffe, 542 F.2d 564, 567, 191 USPQ 429, 431 (CCPA 1976). (citations removed, emphasis added). More recently, the Court further clarified that *In re Mayhew* dealt with a case in which an element was critical in the sense that its omission would result in the invention being wholly inoperative:

> [The dissent] cites *In re Mayhew* for the proposition that "claims failing to recite a necessary element of the invention fail for lack of an enabling disclosure." There, however, the method claims omitted a step without which the invention as claimed was wholly inoperative (meaning it simply would not work and could not produce the claimed product).

Amgen Inc. v Hoechst Marion Roussel Inc. 314 F.3d 1313, 65 USPQ2d 1385, 1403 (CAFC 2003) (emphasis added). Restated equivalently, a rejection as in *In re Mayhew* is properly applied only where the specification as a whole teaches than any embodiment omitting the critical element would be wholly inoperative, i.e., not enabled, and, in particular, is improperly applied if the specification teaches that the omitted feature is merely preferred.

An example of an improper rejection based on an improper determination of a feature as critical is found in *In re Johnson and Farnham*, 558 F.2d 1008, 194 USPQ 187 (CCPA 1977). Therein, the Court overturned a rejection based on the grounds that a

disclosed critical limitation was missing from the claims. The claims at issue were drawn to a chemical polymer. The specification taught that the invention required a minimum "sigma value" for one of the constituent subunits, but the minimum value was not recited in the claims. The examiner had rejected the claims for failing to recite the minimum value on the basis that this minimum value was a critical element of the invention, and the rejection had been upheld by the Board. However, the Court overturned the rejection, stating:

> The PTO would limit appellants to claims reciting a sigma value of at least 0.7. This view is improper because it requires the claims to set forth the practical limits of operation for the invention and it effectively ignores the scope of enablement provided by the specification as a whole.

In re Johnson and Farnham at 195 (USPQ pagination). The Court stated further, "it is the function of the specification, not the claims, to set forth the "practical limits of operation" of an invention" and, further, "[o]ne does not look to claims to find out how to practice the invention they define, but to the specification.". Ibid at 195 (citations omitted).

In summary, a rejection based omission of a critical element is improper unless the specification, taken as a whole, teaches that omission of the element would result in the invention being wholly inoperable. Elements that represent preferred embodiments, e.g., elements that optimize, maximize, or increase accuracy of the results, or elements that set for the practical limits of operation are not critical.

Claims 19-24, 26-33, 35-38, 40-55, and 61-63

Claims 19-24, 26-33, 35-38, 40-55, and 61-63 stand or fall together with respect to the present rejection. Claim 19 is in independent form; claims 20-24, 26-33, 35-38, 40-55, and 61-63 depend directly or indirectly from claim 19. Below, Appellants address each element that represents a reason for the rejection, as summarized, above, and show that the rejection is in error.

Inhibitor of cytokine secretion

The examiner stated that the specification and the post-filing art disclose/teach that the inclusion of an inhibitor of cytokine secretion is essential (last Office action, §5,

page 5). Claim 19 and, thus, claims dependent therefrom recite the inclusion of an inhibitor of cytokine secretion. The use of an inhibitor of cytokine secretion is not an omitted element. Thus, Appellants believe this element does not represent a reason for the rejection.

For completeness, Appellants point out that the specification fully enables the claimed method using an inhibitor of cytokine secretion. As discussed above under Ground I, the use of an inhibitor of cytokine secretion to allow the accumulation of cytokines within activated T cells was known in the art. One of skill in the art, following the teaching of the specification and without undue experimentation, could carry out the claimed methods using a known inhibitor of cytokine secretion.

The use of slant tubes

The rejection was based on the teachings of Example 4, which the examiner alleges teaches that the use of slant tubes is a critical element of the invention. This rejection is in error for any and all of the following reasons.

The specification describes the invention in broad terms omitting the use of slant tubes. For example, the invention is described in the specification omitting the use of slant tubes at page 4, lines 11-15, and in claim 1 as filed. This broad language in the disclosure omitting the allegedly critical features tends to rebut the argument of criticality.

Furthermore, the specification also clearly indicates that the teachings of the examples, including Example 4, are no more than preferred embodiments of the invention. In particular, the specification states at page 13, lines 16-17 with reference to all the examples, "The following examples illustrate certain preferred embodiments of the invention but are not intended to be illustrative of all embodiments" (emphasis added); and at page 18, lines 13-14, "The specific embodiments are given by way of example only,..."). This language in the specification further rebuts the argument of criticality by making clear that the teaching of Example 4 is of preferred embodiments. An element that is merely a preferred embodiment is not a critical feature without which the invention would be wholly inoperative.

Example 4, in distinguishing T cell responses to antigen (Ag) from T cell responses to mitogen and superantigen, states that "the geometry of the T cell/accessory cell interaction was critical for Ag responses; maximal responses were observed in slant tubes...." (page 16, lines 13-14, emphasis added). Appellants point out that the use of slant tubes, which provides one particular geometry of the T cell/accessory cell interaction, is described as maximizing the responses, not as a critical element whose omission would result in the invention being wholly inoperative. An element that merely maximizes responses represents a preferred embodiment and is not a critical feature without which the invention would be wholly inoperative.

For the reasons provided above, Appellants submit that the specification as a whole does not teach that the use of slant tubes is a critical element without which the invention would be wholly inoperative. In fact, the specification clearly describes the use of slant tubes as a preferred embodiment of the invention. An element that is merely a preferred embodiment is not a critical feature without which the invention would be wholly inoperative.

Finally, factual evidence of record demonstrates that the invention is operable, i.e., enabled, without the use of slant tubes. Suni et al., 1998, J. Immunol. Methods. 221:89-98 is Appellants' first publication in a peer-reviewed scientific journal of the methods of the present invention using whole blood samples. Suni et al. include a description of results obtained using the methods of the present invention carried out using culture tubes incubated upright (see §2.2), which demonstrates that the enablement of the invention is not limited to use of the slant tubes. Thus, Suni et al. provide data that demonstrates that the use of slant tubes is not a critical element without which the invention would be wholly inoperative.

The examiner improperly ignored the factual evidence of record on the basis that it was generated post-filing. It is well established that an applicant may provide post-filing data (for example, in a declaration under 37 C.F.R. §1.32) that demonstrates that the written description provided by the specification at the time of filing is, in fact, enabling. Appellants did not cite Suni et al. as augmenting the specification, rather Appellants cited Suni et al. as describing data obtained using the methods of the invention as described in the "summary of the invention" of the specification as filed,

which description does not included the use of slant tubes. Evidence of record that demonstrates that written description provided by the specification at the time of filing is, in fact, enabling must be considered. The examiner erred in ignoring this evidence that demonstrates that the claimed invention, carried out without the use of slant tubes, is enabled and, thus, refutes the allegation that use of slant tubes is a critical element without which the invention would be wholly inoperative. For this additional reason, the allegation that the collection of at least 50,000 events is a critical element of the invention is in error, and the rejection based on this determination is in error.

The number of events collected

The rejection was based on the teachings of Example 4, which the examiner alleges teaches that the use of collection of at least 50,000 events is a critical element of the invention. This rejection is in error for any and all of the following reasons.

The specification describes the invention in broad terms omitting the collection of 50,000 events. For example, the invention is described in the specification omitting the collection of 50,000 events at page 4, lines 11-15, and in claim 1 as filed. This broad language in the disclosure omitting the allegedly critical features tends to rebut the argument of criticality.

Furthermore, the specification also clearly indicates that the teachings of the examples, including Example 4, are no more than preferred embodiments of the invention. In particular, the specification states at page 13, lines 16-17 with reference to all the examples, "The following examples illustrate certain preferred embodiments of the invention but are not intended to be illustrative of all embodiments" (emphasis added); and at page 18, lines 13-14, "The specific embodiments are given by way of example only,..."). This language in the specification further rebuts the argument of criticality by making clear that the teaching of Example 4 is of preferred embodiments. An element that is merely a preferred embodiment is not a critical feature without which the invention would be wholly inoperative.

Example 4 describes that "because of the relatively small size of the Ag-specific populations, accurate assessment of the responses required the routine collection and analysis of at least 50,000 events per determination (page 17, lines 1-2, emphasis added).

Accuracy in the assessment of antigen-specific T-cell frequencies is the statistical problem of distinguishing a small subpopulation of positive events (antigen-specific T cells) from the "noise" of detecting a vastly larger population of negative events. As is well known, the accuracy of a statistical test typically is improved by the collection of a larger data set. Appellants submit that this teaching, which relates to the accuracy of the method, not the basic operability, represents guidance as to the practical limits of operation. It is a function of the specification, not the claims, to set forth the "practical limits of operation" of an invention.

Appellants further submit that an element that affects the accuracy of a method cannot be considered a critical element for claims in which a specific level of accuracy is not a claim element, as is the case here. The claims are drawn to methods of detecting antigen-specific T cells, not to methods having a prerequisite accuracy. Although accuracy may be important for a particular application, such as a commercial application, the level of accuracy is not an element of the claimed invention. Again, teaching that relates to the accuracy of the invention represents guidance as to the practical limits of operation, and it is the function of the specification, not the claims, to set forth the "practical limits of operation" of an invention.

Appellants further point out that Example 3 of the specification describes analyses were carried out using only 48,000 events (page 15, lines 15-16). The description in the specification of the invention using less than 50,000 events clearly demonstrates that the collection of 50,000 events is a not an element whose omission would result in the method being wholly inoperable. Thus, the specification itself clearly refutes the allegation that the collection of at least 50,000 events is a critical element of the invention.

The examiner improperly ignored Example 3 on the basis that it discloses no data (last Office action, page 6, penultimate paragraph). However, Example 3 is not a prospective example; it clearly describes (in past tense) that actual experiments were carried out. It is an error for the examiner to selectively disregard the description in the specification of actual experiments simply because the data were not included - the inclusion of experimental data in the specification is not a requirement under U.S. patent

law. Furthermore, it is an error for the examiner to selectively disregard sections of the specification, as the whole specification must be considered.

For the reasons provided above, Appellants submit that the specification as a whole does not teach that the collection of at least 50,000 events is a critical element without which the invention would be wholly inoperative. In fact, Example 3 clearly refutes the allegation that the collection of at least 50,000 events is a critical element of the invention. Furthermore, the specification clearly describes Example 4 as describing a preferred mode of the invention. An element that merely a preferred embodiment is not a critical feature without which the invention would be wholly inoperative. Furthermore, teaching relating to the accuracy of the invention represents guidance as to the practical limits of operation, and it is the function of the specification, not the claims, to set forth the "practical limits of operation" of an invention. For at least these reasons, the allegation that the collection of at least 50,000 events is a critical element of the invention is in error, and the rejection based on this determination is in error.

Finally, factual evidence of record demonstrates that the invention is operable, i.e., enabled, with fewer events collected. Suni et al. (cited above) describe results obtained using the methods of the invention in which 40,00-50,000 events were collected, which demonstrates that the enablement of the invention is not limited to the collection of at least 50,000 events (see Suni et al., §2.4). Thus, Suni et al. provide data that demonstrates that the collection of at least 50,000 events is not a critical element without which the invention would be wholly inoperative.

Again, the examiner improperly ignored the factual evidence of record on the basis that it was generated post-filing. It is well established that an applicant may provide post-filing data (for example, in a declaration under 37 C.F.R. §1.32) that demonstrates that the written description provided by the specification at the time of filing is, in fact, enabling. Appellants did not cite Suni et al. as augmenting the specification, rather Appellants cited Suni et al. as describing data obtained using the methods of the invention as described in the "summary of the invention" of the specification as filed, which description does not included the collection of at least 50,000 events. Evidence of record that demonstrates that written description provided by the specification at the time of filing is, in fact, enabling must be considered. The examiner erred in ignoring this

evidence that demonstrates that the claimed invention, carried out without the collection of at least 50,000 events, is enabled and, thus, refutes the allegation that use of slant tubes is a critical element without which the invention would be wholly inoperative. For this additional reason, the allegation that the collection of at least 50,000 events is a critical element of the invention is in error, and the rejection based on this determination is in error.

For the reasons discussed herein, and in view of the case law cited, Appellants maintain that claims 19-24, 26-33, 35-38, 40-55, and 61-63 are fully enabled by the specification and that the rejection is in error. Appellants respectfully request that the Board overturn the erroneous rejection of claims 19-24, 26-33, 35-38, 40-55, and 61-63 under the enablement requirement of 35 U.S.C. §112, first paragraph.

Claim 64

Claims 39 and 64 stand separately with respect to the rejection because only a subset of the reasons for the rejection could apply. Claim 39, which depends from independent claim 19, further recites the use of Brefeldin-A, the preferred inhibitor of cytokine secretion exemplified in the specification. Claim 64 corresponds to claim 39 presented in independent form. Thus, the only elements alleged by the examiner to be critical that are not recited in claims 39 and 64 are the use of slant tubes and the collection of at least 50,000 events.

As discussed above, the determination that the use of slant tubes and the collection of at least 50,000 events are critical features of the invention is in error and ignores the teaching of the specification as a whole. whose omission would result in the invention being wholly inoperable. The specification as a whole describes the element as a preferred embodiment. Furthermore, factual evidence of record (Suni et al., supra) provides evidence that the written description in the specification of the invention omitting both the use of slant tubes and the collection of at least 50,000 events, is, in fact, enabling.

For the reasons discussed herein, and in view of the case law cited, Appellants maintain that claims 39 and 64 are fully enabled by the specification and that the

rejection is in error. Appellants respectfully request that the Board overturn the erroneous rejection of claims 39 and 64 under the enablement requirement of 35 U.S.C. §112, first paragraph.

Claim 65

Claim 65 stands separately with respect to the rejection because only a subset of the reasons for the rejection could apply. Claim 65, in addition to reciting all elements of independent claim 19, further recites the use of Brefeldin-A, the preferred inhibitor of cytokine secretion exemplified in the specification, and the use of slant tubes. Thus, the one element alleged by the examiner to be critical that is not recited in claim 65 is the collection of at least 50,000 events.

As discussed above, the determination that the collection of at least 50,000 events are critical features of the invention is in error and ignores the teaching of the specification as a whole. Furthermore, factual evidence of record (Suni et al., supra) provides evidence that the written description in the specification of the invention omitting the collection of at least 50,000 events, is, in fact, enabling.

For the reasons discussed herein, and in view of the case law cited, Appellants maintain that claim 65 is fully enabled by the specification and that the rejection is in error. Appellants respectfully request that the Board overturn the erroneous rejection of claims 39 and 64 under the enablement requirement of 35 U.S.C. §112, first paragraph.

(viii) Claims appendix

1-18. (canceled)

19. A method of detecting T lymphocytes that are specific for a nominal antigen, comprising:

culturing a sample containing peripheral blood mononuclear cells with a nominal antigen;

adding to said sample an inhibitor of cytokine secretion;

permeabilizing said cells;

adding to said sample at least one cytokine-specific antibody and at least one T lymphocyte subset-defining antibody; and then

flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset.

20. The method of claim 19, further comprising the step of adding to said sample, contemporaneously with antigen contact, a costimulus of T cell activation.

21. The method of claim 20, wherein said costimulus is an antibody specific for CD28.

22. The method of claim 20, wherein said costimulus is an antibody specific for VLA-4.

23. The method of claim 19, further comprising contacting said sample with an antibody specific for a T lymphocyte early activation antigen, and then flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset that concurrently bind said early activation antigen-specific antibody.

24. The method of claim 23, wherein said T lymphocyte early activation antigen is CD69.

25. (canceled)

26. The method of any one of claims 19, 20, 23, or 25 wherein said sample is a whole blood sample.

27. The method of claim 26, further comprising the step of adding a cationic chelator after antigen contact is complete but prior to flow cytometric detection.

28. The method of claim 27, wherein said chelator is EDTA or EGTA.

29. The method of claim 28, wherein said chelator is EDTA.

30. The method of claim 26, further comprising the step of lysing red blood cells.

31. The method of claim 19, wherein said nominal antigen is selected from the group consisting of alloantigens, autoantigens, viral antigens, and bacterial antigens.

32. The method of claim 31, wherein said nominal antigen is a viral antigen.

33. The method of claim 32, wherein said antigen is a CMV antigen.

34. (canceled)

35. The method of claim 32, wherein said antigen is a mumps antigen.

36. The method of claim 32, wherein said antigen is a measles antigen.

37. The method of claim 31, wherein said MHC-dependent nominal antigen is a bacterial antigen.

38. The method of claim 37, wherein said antigen is a Mycobacterium tuberculosis antigen.

39. The method of claim 19, wherein said inhibitor of cytokine secretion is Brefeldin A.

40. The method of claim 19, wherein said cytokine-specific antibody is specific for a cytokine selected from the group consisting of: IL-2, IL-4, IL-13, γ-IFN, and TNF-α.

41. The method of claim 40, wherein said cytokine-specific antibody is specific for IL-2.

42. The method of claim 40, wherein said cytokine-specific antibody is specific for IL-4.

43. The method of claim 40, wherein said cytokine- specific antibody is specific for γ-IFN.

44. The method of claim 40, wherein said cytokine-specific antibody is specific for TNF-α.

45. The method of claim 19, wherein said T lymphocyte subset-defining antibody is selected from the group consisting of antibodies specific for: CD3, CD4, CD8, TCR, homing receptors, CD45RO, CD45RA and CD27.

46. The method of claim 45, wherein said T lymphocyte subset-defining antibody is specific for CD3.

47. The method of claim 45, wherein said T lymphocyte subset-defining antibody is specific for CD4.

48. The method of claim 45, wherein said T lymphocyte subset-defining antibody is specific for CD8.

49. The method of any one of claims 19, 20, or 23 wherein said anti-cytokine antibodies, said T lymphocyte subset-defining antibodies, and said early activation antigen-specific antibodies are each conjugated directly to fluorophores.

50. The method of claim 49, wherein said fluorophores are selected from the group consisting of FITC, PE, PerCP, and APC.

51. The method of claim 50, wherein said anti-cytokine antibodies are conjugated to FITC.

52. The method of claim 50, wherein said T lymphocyte subset-defining antibodies are conjugated to PerCP.

53. The method of claim 50, wherein said antibody specific for a T lymphocyte early activation antigen is conjugated to PE.

54. The method of any one of claims 19, 20, or 23 wherein said antigen-contacting step lasts no longer than 24 hours.

55. The method of claim 54, wherein said antigen-contacting step lasts no longer than 6 hours.

56-60. (canceled)

61. The method of claim 19, wherein each of said at least one cytokine-specific antibody is specific for a cytokine selected from the group consisting of IL-2, IL-4, IL-13, IFN-γ, and TNF-α.

62. The method of claim 61, further comprising the step of adding to said sample, contemporaneously with antigen contact, a costimulus of T cell activation, wherein said costimulus is selected from the group consisting of antibodies specific for CD28, VLA-4, CD86, or CD118.

63. The method of claim 61, further comprising contacting said sample with an antibody specific for CD69, and then flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by CD69+ cells in the defined T lymphocyte subset.

64. A method of detecting T lymphocytes that are specific for a nominal antigen, comprising:

culturing a sample containing peripheral blood mononuclear cells with a nominal antigen in the presence of Brefeldin-A;

permeabilizing said cells;

adding to said sample at least one cytokine-specific antibody and at least one T lymphocyte subset-defining antibody; and

flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset.

65. A method of detecting T lymphocytes that are specific for a nominal antigen, comprising:

culturing a sample containing peripheral blood mononuclear cells with a nominal antigen in the presence of Brefeldin-A, wherein said culturing is carried out in a slant tube;

permeabilizing said cells;

adding to said sample at least one cytokine-specific antibody and at least one T lymphocyte subset-defining antibody; and

flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset.

66. **(allowed - not involved in the appeal)** A method of detecting T lymphocytes that are specific for a nominal antigen, comprising:

culturing a sample containing peripheral blood mononuclear cells with a nominal antigen in the presence of Brefeldin-A, wherein said culturing is carried out in a slant tube;

permeabilizing said cells;

adding to said sample at least one cytokine-specific antibody and at least one T lymphocyte subset-defining antibody; and

flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset, wherein said step of flow cytometrically detecting the intracellular binding of said cytokine-specific antibody by cells in the defined T lymphocyte subset comprises analyzing at least 50,000 cells.

(ix) Evidence appendix

None of the evidence submitted pursuant to 37 C.F.R. §§1.130, 1.131, or 1.132 during the prosecution of the present application are being relied upon for the arguments presented herein.

References entered by examiner and relied upon for arguments presented herein are listed below, along with statements setting forth where in the record the evidence was entered in the record by the examiner. Copies of each of these references are provided herein.

Jung et al., 1993, J. Immunol. Methods 159:197-207.

This reference was submitted with the Information Disclosure Statement dated March 8, 1999, and considered by the examiner March 26, 2000 (initialed Form PTO-1449 attached to Office action dated March 27, 2000).

Elson et al., 1995, J. Immunol. 154(9):4294-4301.

This reference was submitted in the Supplementary Information Disclosure Statement dated December 1, 2000, and considered by the examiner on January 22, 2001 (initialed Form PTO-1449 attached to Office action dated January 30, 2001).

Prussin et al., 1995, J. Immunol. Methods 188:117-128.

This reference was submitted with the Information Disclosure Statement dated March 8, 1999, and considered by the examiner March 26, 2000 (initialed Form PTO-1449 attached to Office action dated March 27, 2000)

Picker et al., 1995, Blood 86:1408-1419.

This reference was cited by examiner in the Office action dated September 16, 1997. This reference also was submitted with the Information Disclosure Statement dated March 8, 1999, and considered by the examiner March 26, 2000 (initialed Form PTO-1449 attached to Office action dated March 27, 2000)

Application Note 1: Detection of Intracellular Cytokines in Activated Lymphocytes, Becton Dickinson and Co. (1997): pages 1-12.

This reference was submitted with the Information Disclosure Statement dated March 8, 1999, and considered by the examiner March 26, 2000 (initialed Form PTO-1449 attached to Office action dated March 27, 2000)

Suni et al., 1998, J. Immunol. Methods. 221:89-98.

This reference was submitted with the Information Disclosure Statement dated March 8, 1999, and considered by the examiner March 26, 2000 (initialed Form PTO-1449 attached to Office action dated March 27, 2000). Also provided to the examiner during the interview on August 20, 2003.

Jung et al., 1993, J. Immunol. Methods 159:197-207

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JIM 06608

Detection of intracellular cytokines by flow cytometry

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During the last years it has become increasingly clear that production of most cytokines is not confined to one cell type. Thus, a method to detect cytokines at the single cell level would be a helpful tool to study the contribution of different cells to cytokine production in heterogeneous cell populations. Recently, Sander et al. (1991) demonstrated that it is possible to detect intracellular cytokines by fixation with paraformaldehyde, permeabilization with saponin and subsequent indirect immunofluorescent staining using fluorescence microscopy. Here, we describe a modified method to increase the specific intracellular staining which enables us to detect IFN-γ, IL-2 and IL-4 producing cells by single laser flow cytometry. The carboxylic ionophore monensin was used to interrupt intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex. This resulting increase of the signal/noise ratio permitted us to detect weakly fluorescent cells such as IL-4 producing cells. While IL-4 was detected in approximately 1–3% of peripheral mononuclear cells from healthy donors, up to 30% of the cells produced IFN-γ and nearly 50% IL-2 after phorbol ester and ionomycin stimulation. Microscopic and flow cytometric analysis showed a highly significant correlation. Using three-color flow cytometry it was possible to measure intracellular cytokines and cell surface markers simultaneously. Subpopulations of human T cells (e.g., $CD4^+$ $CD45RO^-$) producing a restricted cytokine pattern could be identified by cell surface staining and were characterized by their cytokine production. Consequently, there was no further need for cell sorting to determine cytokine producing subsets in heterogeneous cell populations. We have tested human T cell clones for intracellular cytokine production and found a high concordance to ELISA analysis of the supernatants. We conclude that detection of intracellular cytokines by flow cytometry is a rapid, easy and semiquantitative assay which may be used to study individual cells in heterogeneous populations as well as to screen homogeneous cells for their cytokine pattern. This method is particularly relevant in view of the accumulating evidence of the functional role that subsets of (T) cells may play in various diseases depending on the pattern of cytokines they produce.

Key words: Flow cytometry; Cytokine; Monensin; T cell clone; Intracellular immunofluorescence

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Abbreviations: FACS, fluorescence activated cell sorter; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; PBMC, peripheral mononuclear cells; PFA, paraformaldehyde; IFN, interferon; IL, interleukin; FITC, fluoresceinisothiocyanate; PE, phycoerythrin; PerCP, peridininchlorophyll; Th, T helper; TCC, T cell clone; ConA, concanavalin A; RITC, rhodamineisothiocyanate; AMCA, 7-amino-4-methylcoumarin-3-acetate; CD, cluster of differentiation; PI, propidium iodide; HBSS, Hanks' balanced salt solution.

Introduction

Cytokines are important mediators during immune responses and are produced by a variety of activated cells. The production of cytokines by activated peripheral blood cells has been studied at the protein level using bioassays and monoclonal antibody techniques and at the mRNA level. The use of such bulk-release detection methods often assumes that, under any condition, cells of a given phenotype show identical or similar levels of cytokine secreting behavior. However, such techniques can yield ambiguous results since it is often difficult to ensure that a given cell population is free of contaminating cells which may secrete the same cytokine. Moreover, since cytokines act as mediators of cell-cell communication, relevance is assigned to the local production of cytokines by individual cell populations. These considerations have prompted the evaluation of various strategies for studying cytokine production at the single cell level which are reviewed by Lewis (1991). An ideal technique should identify producer cells by cell surface markers and by cytokine(s) production simultaneously. Intracytoplasmic staining of cytokines and analysis by flow cytometry seems to fulfill these requirements.

However, there are limitations from the methodological as well as from the theoretical point of view. Definitive studies correlating the synthesis and intracellular storage of cytokines with their subsequent release have yet to be performed. In addition, staining of intracytoplasmic cytokines often results in weak signals which can hardly be discriminated from background staining.

In this report we show that these problems can be circumvented by the use of monensin which inhibits intracellular traffic pathways leading to an accumulation of proteins and subsequently to an enhanced fluorescent signal.

Materials and methods

Antibodies and reagents

Anti-IFN-γ (mouse IgG1, GZ-4 (Boehringer Mannheim, Germany), mouse IgG2a (Genzyme, Boston, USA)), anti-IL-2 (mouse IgG2a, N7.48A) kindly provided by A. Jurado (Sandoz, Basel), anti-IL-4 (mouse IgG1, 8F12, 4D9, 1G1) generated at Ciba Geigy, Basel (see also Andersson et al., 1990).

Isotype-specific goat anti-mouse IgG1 or IgG2a second step antibodies conjugated to FITC, PE, RITC or biotin were obtained from Southern Biotechnology (Birmingham, AL, USA). TRI-Color conjugated goat anti-mouse IgG1 and streptavidin were obtained from Medac (Hamburg, Germany), streptavidin-PE from Serva (Heidelberg, Germany), streptavidin-AMCA (Jackson Immunoresearch, Baltimore, MD, USA). Control antibodies for IgG1 and IgG2a were purchased from Coulter (Krefeld, Germany), mouse IgG and goat IgG from Sigma (St. Louis, MO, USA). The following antibodies against surface antigens, pure or labeled with FITC, PE or PerCP, were kindly provided by Becton Dickinson (Heidelberg, Germany): anti-CD4, anti-CD8, anti-CD45R0, anti-CD45RA, anti-CD3, anti-CD16, anti-CD56, anti-CD14, anti-CD20.

Natural IFN-γ and IFN-β (kindly provided by Bioferon, Laupheim, Germany), recombinant IL-2 and IL-4 (Genzyme, Boston, MA, USA).

Phorbol 12-myristate 13-acetate (PMA), ionomycin, propidium iodide (PI), dimethylsuberimidate and monensin were obtained from Sigma, phytohemagglutinin (PHA) from Biochrom (Berlin, Germany), saponin and paraformaldehyde (PFA) from Riedel de Haen (Seelze, Germany), hepes buffer from Serva (Heidelberg, Germany), HBSS buffer from Biochrom (Berlin, Germany).

Cells and cell culture

Peripheral blood was obtained from healthy human volunteers. Mononuclear cell (PBMC) were isolated by Ficoll gradient centrifugation (Biochrom, Berlin, Germany). For purification of T cells or further cell sorting, PBMC were incubated with neuraminidase treated sheep red blood cells (Behringwerke, Marburg, Germany) and centrifuged over Ficoll. Erythrocytes were eliminated by ammonium chloride lysis. The purity of such T cells was greater than 95% as assessed by flow cytometry, and these cells are subsequently referred as T cells. For isolation of memory or

naive cells, T cells were incubated with a cocktail of antibodies containing, anti-CD16, anti-CD56, anti-CD20, anti-CD14, (anti-CD8) and anti-CD45RA or anti-CD45R0. Cell sorting was done by MACS according to Miltenyi et al. (1990) using rat anti-mouse IgG1 or IgG2a antibodies labeled with superparamagnetic beads (Miltenyi, Bergish Gladbach, Germany). Depleted cells were highly enriched for $CD4^+$ ($CD3^+$) $CD45R0^+$ or $CD4^+$ ($CD3^+$) $CD45R0^-CD45RA^+$ cells (> 95%) and are referred to as memory-cells ($CD4^+CD45R0^+$) and naive-cells ($CD4^+CD45R0^-$ $CD45RA^+$) respectively. Only depleted cells were used for experiments.

Cells ($2 \times 10^5/100\ \mu l$) were cultured in 96 well flat bottom plates or in 24 well plates (2×10^6/ml) (Nunc, Wiesbaden, Germany) for various periods of time at 37°C and 8% CO_2 in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicilline, 100 μg/ml streptomycin, 2×10^{-5} M mercaptoethanol (Biochrom) and 10% AB serum (Sigma). Cells were stimulated with PMA 1–10 ng/ml + 1 μM ionomycin, PHA 2.4 μg/ml or PHA 2.4 μg/ml + PMA 1 ng/ml in the presence or absence of monensin as indicated.

Allergen-specific T cell clones (TCC) were obtained as described (Sager et al., 1992). Briefly, from two patients with atopic dermatitis a punch biopsy specimen was obtained from inflamed skin exposed to *Dermatophagoides pteronyssinus* (Derp) (Bencard, Neuss, Germany). Isolated T cells were cloned using the limiting dilution assay with Derp (1 μg/ml) and irradiated autologous PBMC as antigen presenting cells. Responder T cells were expanded with allogeneic irradiated PBMC as feeder cells, PHA and rIL-2. After 8–10 days of expansion, cloned cells (10^6/ml) were stimulated with ConA (5 μg/ml) (Sigma). Supernatants were collected after 24 h for the assessment of IL-4 and IL-2 and after 48 h for IFN-γ. To determine the number of cells staining positive for intracellular IFN-γ, IL-2 and IL-4, cells were harvested after 6 h of restimulation in the presence of 3 μM monensin.

Staining

Cultured cells were washed twice in HBSS and then fixed in ice cold HBSS containing 4% paraformaldehyde (PFA) for 10 min. After two further washes in HBSS the cells were resuspended to 2×10^5 in 300 μl HBSS containing 0.1% saponin, 10% AB serum, 100 μg/ml goat IgG and 0.01 M Hepes buffer. After 10 min, the cells were spun down and cytokine specific antibodies diluted in HBSS with 0.1% saponin and 0.1 M Hepes buffer (saponin buffer) were added at a concentration of 1 μg/ml for 30 min at room temperature. Cells were washed twice in saponin buffer and subsequently incubated with isotype-specific second step antibodies in a concentration ranging from 0.5 to 5 μg/ml for 20 min in the dark. Cells were washed in saponin buffer and stained with streptavidin conjugates or in the case of surface staining incubated with 200 μg/ml mouse IgG diluted in saponin buffer for 15 min. After subsequent washing in saponin buffer cells were washed twice in HBSS and stained for 20 min with different monoclonal antibodies in order to determine their surface phenotype. As a last step, cells were washed in HBSS.

To determine the percentage of dead cells and the effects of monensin on surface antigens, cultured cells were not fixed but washed in HBSS containing sodium azide 0.1%. PI was added after surface staining at a concentration of 10 μg/ml.

ELISA

For the measurement of cytokines in the supernatants of activated T cells or T cell clones the following commercially available ELISA kits were used: IFN-γ (Biomar, Marburg, Germany and Holland Biotechnology, Leiden, Netherlands), IL-4 (Genzyme, Boston, USA and Research and Diagnostic Systems, purchased from Biermann, Bad Nauheim, Germany), IL-2 (T Cell Science, Biermann, Bad Nauheim, Germany).

Flow cytometry and fluorescence microscopy

A FACScan flow cytometer (Becton Dickinson, Mountain View, USA) equipped with a 15 mW argon ion laser and filter settings for FITC (530 nm), PE (585 nm) and TRI-Color (Medac, Hamburg, Germany) or PerCP (Becton Dickinson, USA) emitting in the deep red (> 650 nm) was used. 5000–10,000 cells were computed in list

mode and analyzed using the FACScan research software (Becton Dickinson).

Fluorescence microscopy was performed with a UV microscope (Olympus, Hamburg, Germany) equipped with a 100 W mercury arc lamp and filters for detection of AMCA (340 nm), FITC (525) and RITC (580 nm).

Results

Monensin increases the signal / noise ratio and the number of detectable stained cells

Human peripheral blood mononuclear cells (PBMC) were activated with PMA and ionomycin in the presence or absence of monensin and stained for intracellular cytokines. As shown in Fig. 1 monensin increased the signal/noise ratio by enhancing the fluorescence intensity as well as increased the number of detectable stained cells. Ten hours after stimulation, nearly 50% of the lymphocytes were stained positive for IL-2 in the presence of monensin while only 11% positive cells were seen in the absence of monensin. IL-4 producing cells in the peripheral blood of healthy humans are rare (Andersson et al., 1990) and thus – with this method – their detection was improved when monensin was added to the culture. But the mean fluorescence intensity for IL-4 was never as high as for IL-2 or IFN-γ. The kinetics of IFN-γ and IL-2 production in PBMC after PMA and ionomycin stimulation shown in Fig. 2 demonstrate that in the presence of monensin more positive cells were detected (50% vs. 18% for IL-2, 25% vs. 12% for IFN-γ) and the peak of production was delayed indicating an accumulation of the cytokine in the cell (10 h vs. 8 h for IL-2, 6 h and 15 h vs. 6 h for IFN-γ). Maximal IL-4 production was detected after 2 h of stimulation (1–3%) and absent after 8 h and longer (data not shown).

We found an inverse relationship between the number of activated T cells stained for cytokines and the amount of secreted protein in the supernatants of these cells in the presence or absence of monensin (Table I). This suggests that protein secretion is markedly inhibited by monensin.

To determine the optimal concentration of monensin while avoiding toxicity, we tested the compound over the concentration range 10 nM to 100 μM and determined the percentage of dead cells by propidium iodide (PI) staining after 6 and

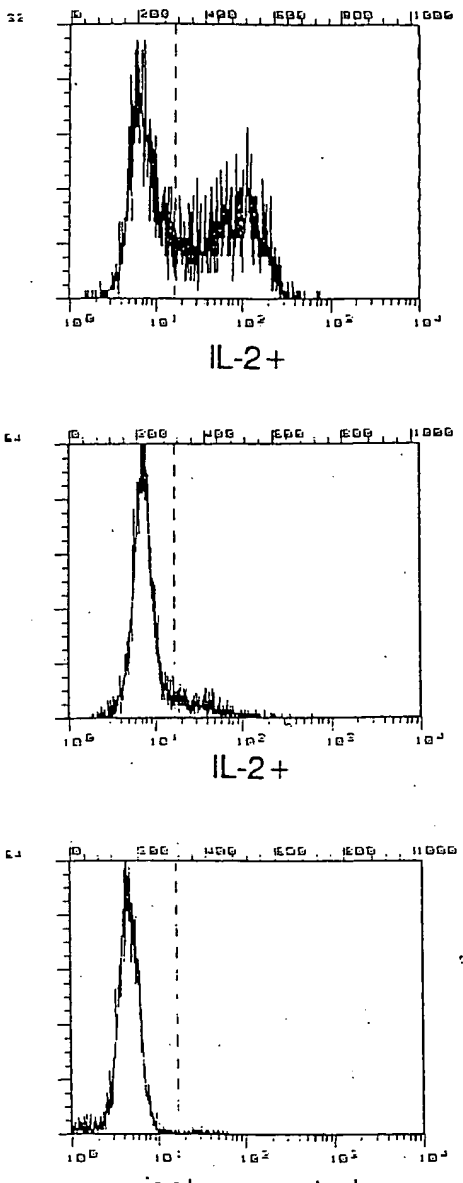

Fig. 1. Effect of monensin on intracellular IL-2 staining. PBMC were stimulated with PMA 5 ng/ml + 1 μM ionomycin in the presence (top) or absence (middle) of 3 μM monensin for 10 h and stained for intracellular IL-2. An analysis gate was set on lymphocytes. 50% of lymphocytes were positive in the presence and 11% in the absence of monensin. A control using an irrelevant mouse IgG2a antibody showed <1% non-specifically stained cells (bottom).

TABLE I

EFFECT OF MONENSIN ON INTRACELLULAR AND EXTRACELLULAR CYTOKINE CONTENT [a]

Monensin 3 μM	ELISA IFN-γ U/ml	FACS IFN-γ % T cells	ELISA IL-4 pg/ml	FACS IL-4 % T cells
Exp. 1				
without	44.8	11.2	2072	0.5
with	17.8	36.7	208	3.0
Exp. 2				
without	106.6	12.4	2403	1.0
with	22.6	22.4	142	1.8
Exp. 3				
without	62.7	10.3	3100	1.3
with	17.2	24.5	359	3.0
Exp. 4				
without	38.1	8.2	277	0.6
with	11.9	27.3	< DL [b]	0.4

[a] Peripheral T cells from four healthy donors were stimulated with PMA 1 ng/ml + 1 μM ionomycin with or without 3 μM monensin. Intracellular staining (FACS) was done after 6 h, supernatants were harvested after 18 h for ELISA analysis.
[b] Detection limit.

18 h of culture. In addition, positive cells for IFN-γ and IL-4 and the amount of protein secreted into the supernatants were assessed. As shown in Table II we found a marked toxic effect of monensin at a concentration of 100 μM even after 6 h of culture, while concentrations < 1 μM did not induce significant cell death. Monensin at a concentration of 1 μM was highly toxic after 24 h (> 60% PI positive cells). After 48 h nearly all cells were dead (data not shown). Interestingly, 10 nM monensin was effective in reducing protein secretion and in enhancing intracellular staining. Because cytokine expression was of interest in combination with cell surface staining, we tested the influence of monensin on a variety of common cell surface antigens (CD3, CD4, CD8, CD20, CD45R0, CD14). PBMC from a single donor were stimulated with PMA plus PHA for 15 h, dead cells were excluded from analysis by PI staining. When monensin was used at various concentrations (0.01–100 μM), membrane marker expression was not found to vary by more than 12.4% and on average was within 5.1% of the control. We have performed three further experiments using a concentration of 1 μM monensin and have not found any significant alterations in cell surface marker expression after 6 and 18 h (data not shown). Thus, in further experiments we used monensin at a concentration of 0.5 = 5 μM for a period not longer than 15 h.

Fixation and permeabilization

In accordance with Sander et al. (1991), we used 4% paraformaldehyde (PFA) to fix and 0.1% saponin to permeabilize the cells. Following this treatment the scatter properties of the cells was retained and surface antigenicity was not changed

TABLE II

INFLUENCE OF MONENSIN ON INTRACELLULAR CYTOKINE STAINING, EXTRACELLULAR PROTEIN CONTENT AND TOXICITY [a]

Monensin μM	Toxicity [b] PI % PBMC		FACS IFN-γ % lymphocytes	ELISA IFN-γ U/ml	FACS IL-4 % lymphocytes	ELISA IL-4 pg/ml
	6 h	18 h	6 h	18 h	6 h	18 h
Without	1.3	5.1	4.9	13.0	1.5	140
0.01	1.7	6.8	9.4	7.0	1.7	97
0.1	1.6	10.8	11.6	7.7	2.4	116
1.0	1.8	14.0	13.4	7.9	3.8	112
10	3.2	26.7	16.7	9.3	4.7	83
100	9.6	36.9	14.2	9.3	2.8	48

[a] PBMC were cultured with PMA 1 ng/ml + 1 μM ionomycin in the presence or absence of the indicated concentrations of monensin. Intracellular staining (FACS) was performed after 6 h, supernatants were harvested after 18 h for ELISA analysis. Results are expressed as mean of three experiments.
[b] Dead cells were determined by propidium iodide (PI) staining of unfixed cells after 6 and 18 h and expressed as percentage of all cells. Mean of three experiments.

(Sander et al., 1991; Jacob et al., 1991). However, PFA treatment enhanced autofluorescence and was not suitable for every antibody, in agreement with the findings of Sander et al. (1991). Using selected antibodies, lower concentrations of PFA – even 0.25% – were useful for the detection of IFN-γ and IL-2 producing cells but the number of stained cells was reduced in comparison to 4% PFA. Furthermore, scatter properties were altered. A higher concentration resulted in a dramatic increase in autofluorescence. In a search for an alternative fixation method we tested ethanol and methanol but could not detect a positive signal. Only the dimidoester, dimethylsuberimidate (Hassell and Hand, 1974) was useful; however, the staining resulted in a high background and the results were not as favourable as PFA treatment.

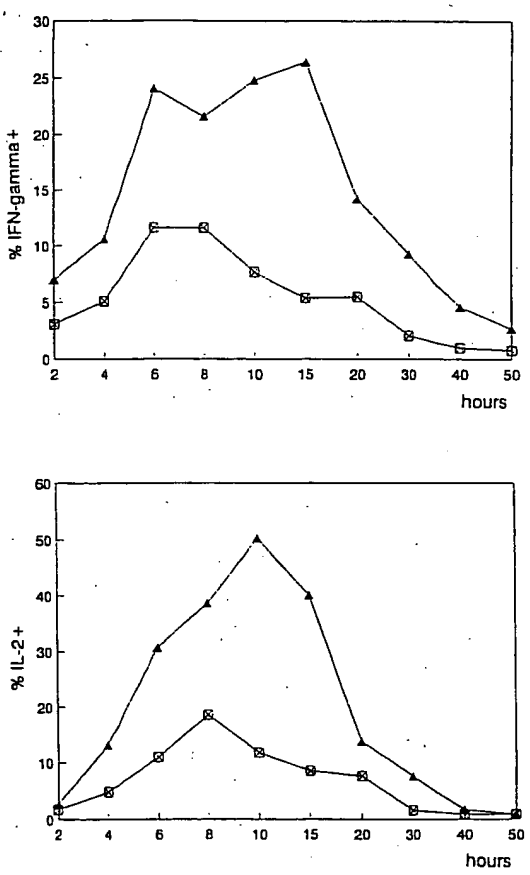

Fig. 2. Kinetic analysis of intracellular IFN-γ and IL-2 production. PBMC were stimulated with PMA 1 ng/ml + 1 μM ionomycin in the presence (filled triangle) or absence (open squares) of 1 μM monensin, harvested at the indicated time points and analyzed for intracellular IFN-γ and IL-2 production. Monensin was present from the beginning of the culture for 15 h. For the following time points (20–50 h) monensin was added 10 h before harvesting. An analysis gate was set on lymphocytes.

Specificity of intracellular staining

To evaluate the specificity of anti-cytokine antibodies, competition experiments were performed. $CD4^+CD45R0^+$ memory cells activated with PMA and ionomycin were double stained for intracellular IFN-γ and IL-4 production in the absence or presence of increasing concentrations (10^2–10^5 U/ml) of natural IFN-γ. As shown in Fig. 3 IFN-γ staining was completely blocked with 10^5 U/ml natural IFN-γ while 10^5 U/ml natural IFN-β had no effect. Furthermore, the simultaneous detection of IL-4 producing cells was not influenced by natural IFN-γ or IFN-β addition.

Intracellular staining correlated with cytokine patterns of human T cell clones as evaluated by ELISA analysis

We have tested nine human allergen-specific T cell clones (TCC) for intracellular cytokine production and compared the results with ELISA analysis of culture supernatants. TCCs were stimulated with ConA (5 μg/ml) in the presence of monensin (3 μM) for 6 h. Table III shows that intracellular staining correlated well with the restricted cytokine production of the cloned cells.

Surface bound cytokines and cytokine uptake

Flow cytometric analysis is not useful to distinguish between signals from the interior or the surface of a cell. In order to determine the percentage of cells expressing a cytokine on the surface membrane, cultured cells were stained for IFN-γ, IL-2 or IL-4 in a buffer free of saponin. This was important because saponin has an effect even at a concentration of 0.001%. Fixation with PFA alone did not allow antibodies to penetrate the membrane. In three experiments we found a small but significant proportion of cells which were positive for IFN-γ or IL-2 (3–9%), while IL-4 could not be detected on the cell surface

after 6 h of culture with PMA and ionomycin. This was independent of fixation. As expected, monensin treatment (1–3 μM) resulted in a slightly reduced number of IFN-γ or IL-2 membrane positive cells (3–6%) indicating again that intracellular transport was inhibited.

To exclude the possibility that intracellular signals are in part due to an uptake of secreted proteins, we added natural IFN-γ (1000 U/ml) or rIL-2 (200 U/ml) or rIL-4 (200 U/ml) to the culture medium and tested peripheral T cells after 6 h of activation in the presence or absence of monensin. We could not detect an increased number of positive cells irrespective of monensin treatment. This was the case for membrane bound as well as for intracellular staining.

Detection of a restricted cytokine production of T cell subsets by three-color flow cytometry

To determine the cytokine pattern of a subpopulation of cells by conventional means it is necessary to isolate these cells and to measure the cytokine production by ELISA, bioassay or mRNA expression. We investigated whether it is possible by three-color FACS analysis to define a restricted cytokine pattern of a T cell subset without prior cell sorting using cytokine and cell surface staining simultaneously. As already shown, human naive ($CD4^+CD45RA^+CD45R0^-$) and memory ($CD4^+CD45R0^+CD45RA^-$) cells differ in their ability to synthesize cytokines. While naive cells are restricted for IL-2 production, memory cells produce the whole pattern of

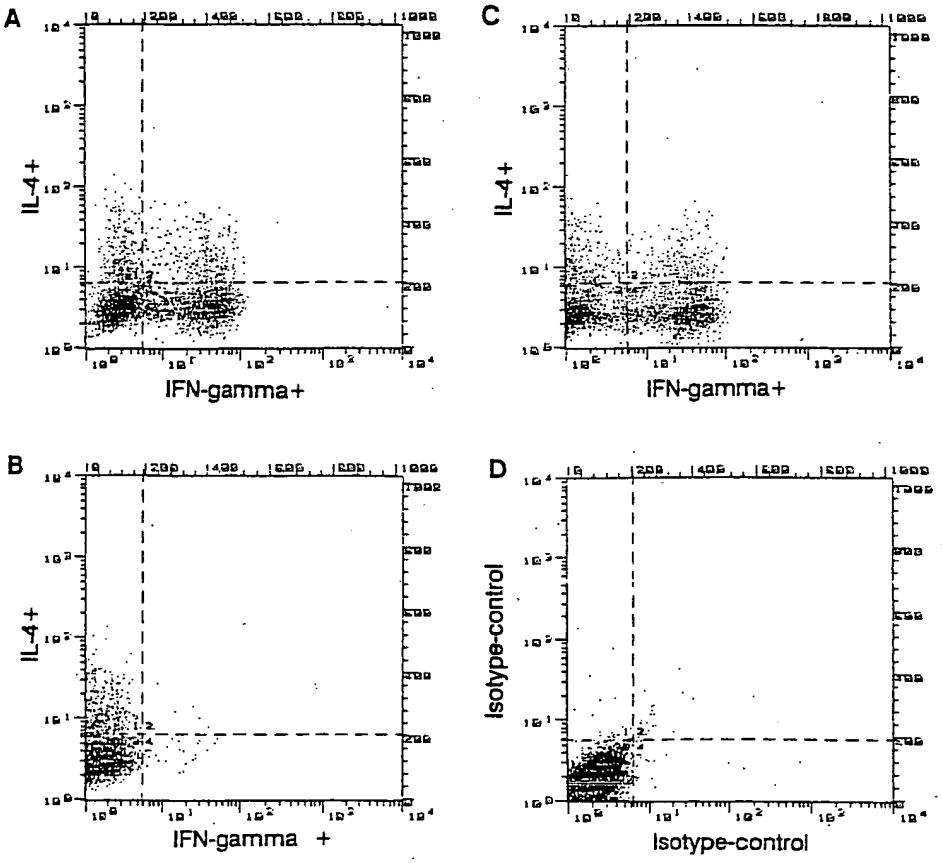

Fig. 3. Specificity of intracellular cytokine staining. $CD4^+$ $CD45R0^+$ cells were isolated from peripheral blood as described and cultured with PMA 5 ng/ml + 1 μM ionomycin in the presence of 3 μM monensin for 6 h. Cells were double stained for IFN-γ and IL-4 (*A*). 100,000 U/ml natural IFN-γ blocked the binding of the anti-IFN-γ antibody (*B*) while 100,000 U/ml IFN-β had no effect (*C*). Plot *d* shows an isotype control with irrelevant mouse IgG1 and mouse IgG2a antibodies.

T cell cytokines (Andersson et al., 1990; Akbar et al., 1991; Ehlers and Smith, 1991). Thus, we sorted naive and memory cells as described and investigated intracellular IL-2, IFN-γ and IL-4 production. These results were compared to isolated $CD4^+$ T cells or $CD3^+$ T cells, which were stained with the surface marker anti-CD45R0 and anti-cytokine antibodies.

Analysis was performed using a gate for $CD45R0^+$ or $CD45R0^-$ cells. Table IV shows the comparison between sorted memory ($CD4^+$ $CD45R0^+$) and naive ($CD4^+$ $CD45RA^+$ $CD45R0^-$) cells with isolated $CD4^+$ T cells from the peripheral blood of a single donor. Activated cells were stained for IL-4 and IFN-γ, the isolated $CD4^+$ T cells were further stained with anti-CD45R0. Both techniques were able to detect restricted IL-4 and IFN-γ production in the $CD45R0^+$ subpopulation. Furthermore, we used sorted $CD3^+$ $CD45R0^+$ and $CD3^+$ $CD45R0^-$ cells and compared intracellular IL-2 and IFN-γ production with isolated T cells stained with anti-CD45R0. The analysis shown in Table IV demonstrates again that both methods yield identical results: IL-2 producing cells were found in both cell populations, while IFN-γ producers are restricted to the CD45R0 phenotype. Thus, the method permits determination of the cytokine pattern of a subpopulation of cells within a mixture of different cells.

FACS analysis of intracellular cytokines reveales an excellent correlation with microscopic examination

To ensure that the flow cytometric signal is of cytoplasmic origin and to compare FACS and

TABLE III

INTRACELLULAR PRODUCTION AND SECRETION OF CYTOKINES BY HUMAN ALLERGEN-SPECIFIC T CELL CLONES

Clone [a]	Detection	IL-2	IL-4	IFN-γ	Accordance [d]
LMS II-D1	ELISA [b]	< DL [e]	> 2	< DL	
	FACS [c]	0	17.6	0	+ + +
LMS II-E7	ELISA	0.09	0.3	> 5	
	FACS	2.1	2.1	9.5	+ + +
LMS II-E15	ELISA	0.3	> 2	1.2	
	FACS	1.2	38.5	0.6	+ +
LMS II-E5	ELISA	> 5	> 2	> 5	
	FACS	9.9	45.8	3.9	+ + +
DK-E1	ELISA	< DL	< DL	> 5	
	FACS	2.7	2.1	14.1	+
HS-E13	ELISA	0.025	> 2	> 5	
	FACS	1.5	24.7	17.1	+ + +
HS-E22	ELISA	< DL	0.68	> 5	
	FACS	0	8.4	4.9	+ + +
HS-E31	ELISA	0.425	> 2	> 5	
	FACS	5.1	62.2	30.6	+ + +
HS-E33	ELISA	< DL	> 2	> 5	
	FACS	1.6	49.2	22.6	+ +

[a] T cell clones were stimulated with ConA (5 μg/ml) after 8 days of expansion with feeder cells, PHA and rIL-2.
[b] Supernatants were harvested after 24 h to detect IL-4 and IL-2 and after 48 h for IFN-γ (without monensin). ELISA results are shown in ng/ml.
[c] Intracellular staining (FACS) in percent of all cells after 6 h of culture in the presence of 3 μM monensin.
[d] Correlation of results obtained by both methods: +(one cytokine), + +(two cytokines), + + +(three cytokines).
[e] Detection limit.

TABLE IV

SIMULTANEOUS DETECTION OF TWO INTRACELLULAR CYTOKINES IN T CELL SUBPOPULATIONS IDENTIFIED EITHER BY CELL SORTING OR BY GATING [a]

Subset [b]	Cytokine	Sorted [c] % of cells	Gated [d] % of cells
$CD4^+ CD45R0^+$	IFN-γ^+	40.8	41.6
	IL-4^+	8.4	7.6
	IFN-γ^+ IL-4^+	3.5	3.8
$CD3^+ CD45R0^+$	IL-2^+	42.7	45.5
	IFN-γ^+	23.1	39.6
	IL-2^+ IFN-γ^+	13.3	18.3
$CD4^+ CD45R0^-$	IFN-γ^+	2.0	9.8
	IL-4^+	< 1	< 1
	IFN-γ^+ IL-4^+	< 1	< 1
$CD3^+ CD45R0^-$	IL-2^+	29.7	25.9
	IFN-γ^+	1.8	6.3
	IL-2^+ IFN-γ^+	0.7	2.3

[a] Cells were cultured with PMA 5 ng/ml+1 μM ionomycin in the presence of 3 μM monensin for 6 h.

[b] For the $CD4^+$ experiment, gated and sorted cells were obtained from the same donor, while the CD^{3+} experiment was performed with cells from different donors.

[c] T cell subpopulations were isolated from human blood as described and stained for intracellular IFN-γ plus IL-4 (double fluorescence) and IFN-γ plus IL-2, respectively.

[d] $CD4^+$ ($CD3^+$) T cells were isolated from human blood as described. After intracellular cytokine staining cells were incubated with an anti-CD45R0 antibody (triple fluorescence). An analysis gate was set on the $CD45R0^+$ or on the $CD45R0^-$ ($CD45RA^+$) population.

microscopically derived data, we investigated ten samples for IL-2, IFN-γ and anti-CD45R0 staining by flow cytometry as well as by fluorescence microscopy. Using the Spearman test we found a highly significant correlation for IFN-γ (with monensin $r = 1.0$, without monensin $r = 0.94$) and for IL-2 (with monensin $r = 0.92$, without monensin $r = 0.89$) which is comparable to a previous report from Andersson et al. (1988).

Discussion

In this report we describe the use of monensin to improve the detection of cytokine producing cells by flow cytometric analysis. This procedure permitted detection of intracellular IFN-γ, IL-2, and IL-4. Monensin is a lipophilic metabolite of *Streptomyces cinnamonensis* which binds Na^+, K^+ and H^+ ions, leading to the disruption of ion gradients in biological membranes (Tartakoff, 1983). This results in a perturbation of the Golgi complex and an interruption of transport to the cell membrane without influencing protein de novo synthesis. As shown, monensin treatment results in an accumulation of a cytokine in the cell, leading to an enhanced number of positively stained cells after a definite time period and, consequently, to a delayed peak in intracellular cytokine production. Fluorescence intensity under monensin treatment was markedly increased, which is in agreement with reports from other groups (Lee et al., 1990; Henter et al., 1988). Even at low concentrations (0.01 μM) monensin was active. The concentrations used in this study (0.5–3 μM) were not toxic for cells using culture time up to 15 h. Furthermore, monensin did not alter cell surface markers, thereby permitting simultaneous detection of intracellular and membrane antigens.

Although monensin treatment resulted in an improvement of the technique, there are still some limitations to note. From a variety of tested fixation methods we have chosen PFA treatment, although some anti-cytokine antibodies were not suitable possibly due to the different affinities for their epitopes or to alterations of some antigenic sites. In combination with PFA fixation, permeabilization of the cell membrane with saponin was preferred because, in contrast to Tween 20 or Triton X-100 (data not shown), it did not change the scatter properties and as already shown by Jacob et al. (1991) it did not alter most surface markers. We incubated the cells with mouse IgG after intracellular staining and before membrane staining in order to block the free binding sites of the second stage antibodies. This procedure enabled us to work with anti-cytokine antibodies of two different isotypes and with any antibody for staining surface antigens. Alternatively, it was possible to detect one cytokine and two surface structures. New fluorescent dyes emitting beyond 650 nm are now available making three-color flow cytometry possible. The use of TRI-Color (a tandem containing R-phycoerythrin and cyanin 5) conjugated second

step antibodies or streptavidin permitted us to detect subpopulations of peripheral T cells with restricted cytokine patterns. Detection of more than one cytokine by indirect immunofluorescence techniques needs highly isotype specific second step antibodies. Those used in this study were selected in this respect but the availability of directly conjugated antibodies would permit circumvention of this problem.

We could demonstrate that the intracellular signal, in the case of IFN-γ was highly specific as demonstrated by blocking the staining with large amounts of the particular cytokine. Similar results were seen for IL-4 using a transfected cell line and the same anti-IL-4 antibodies as used in our study (Andersson et al., 1990). Whereas in unstimulated cells, membrane or intracellular cytokines were never observed, a few cells with membrane bound cytokines could be detected after stimulation, indicating that monensin treatment could not completely interrupt the transport systems for the cytokines. However, such cells (IL-2 and IFN-γ membrane positive) mostly had a lower fluorescence intensity than the intracellular stained cells. Furthermore, we found no evidence that detection of an intracellular cytokine was the result of internalization. Thus, the fluorescent signal represents cytokine production.

Nine human T cells clones were tested for cytokine production by intracellular FACS analysis and compared to ELISA analysis of the supernatants. We found a high correlation between the two detection methods indicating that intracellular staining was suitable for identifying homogeneous T cells with respect to the cytokine pattern (Th0, Th1 or Th2) they produce. Thus, detecting two or three cytokines simultaneously may allow one to characterize antigen specific T cells without prior cloning. Not only the number of stained cells but also differences in fluorescence intensity may reflect the net amount of secreted cytokines. Secretion of more than 2 ng/ml IL-4 was associated with 17% or more IL-4 positive cells which had a weak fluorescence intensity. In contrast, less than 10% IL-2 or IFN-γ positive cells having a much higher fluorescence intensity produced more than 5 ng/ml protein in the supernatants.

PMA + ionomycin or PHA + PMA stimulation were used as potent stimuli to induce cytokine production when establishing the method. With PHA alone, the number of cytokine positive cells was lower. Other studies have already shown that stimulation with anti-CD3 (Andersson and Sander, 1989), tetanus toxoid (Kabilan et al., 1990) or staphylococcal enterotoxin B (Lee et al., 1990) induced IFN-γ or IL-2 in amounts which were detectable by intracellular fluorescence microscopy. Thus, the method is sensitive enough to measure cytokine production under different conditions of stimulation.

The described method is quick (2–6 h of culture, 2–3 h staining and analysis), easy and sensitive enough to require only a few cells (10^5) per test. Fixed and stained cells can be stored in the dark for at least one day for IL-4 and 3 days for IL-2 and IFN-γ. Although the method is a semiquantitative assay we believe that applications may include characterization of cytokine production of immunocompetent cells within heterogeneous cell populations, screening of cytokine patterns in homogeneous cell populations and functional characterization of cytokine producing cells in various diseases such as autoimmunity, allergy and after immunosuppressive therapy.

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Flow Cytometric Analysis for Cytokine Production Identifies T Helper 1, T Helper 2, and T Helper 0 Cells Within the Human $CD4^+CD27^-$ Lymphocyte Subpopulation

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Using three-color flow cytometric analysis for the detection of intracellular cytokines, we have been able to determine the exact combination of cytokines produced by individual T lymphocytes. Because $CD4^+CD27^-$ lymphocytes have been shown to produce more IL-4 and IL-5 than $CD4^+CD27^+$ lymphocytes, cells from normal individuals ($n = 4$) and helminth-infected patients ($n = 4$) were sorted magnetically for the $CD4^+CD27^+$ and the $CD4^+CD27^-$ subpopulations. Intracellular staining for IL-4, IL-5, and IFN-γ subsequent to mitogen stimulation for 6 h revealed that although almost no $CD4^+CD27^-$ lymphocytes produce both IL-5 and IFN-γ (0.03–1.4%), a distinct proportion produce both IL-4 and IFN-γ (0.1–8.0%), and 66% to 84% of IL-5-producing cells also produce IL-4. Patients and normal individuals had the same functional T cell subsets, but the $CD4^+CD27^-$ lymphocytes from patients had higher frequencies of cells producing IL-4 (geometric mean (GM), 24.3% vs 16.4%) or IL-5 (GM, 10.2% vs 2.9%), whereas those of normal individuals had higher frequencies of cells producing IFN-γ (GM, 44.5% vs 17.2%; $p = 0.043$). These analyses also revealed that the $CD4^+CD27^-$ population included significantly higher frequencies of cells that were $IL\text{-}5^+IFN\text{-}\gamma^-$ (GM, 4.9% vs 1.5%; $p = 0.025$), $IL\text{-}4^+IFN\text{-}\gamma^-$ (GM, 13.8% vs 3.5%; $p = 0.025$), and $IFN\text{-}\gamma^+IL\text{-}4^-IL\text{-}5^-$ (GM, 27.3% vs 12.0%; $p = 0.011$) than the $CD4^+CD27^+$ population. Thus, we have clearly demonstrated Th1, Th2, and Th0 cell subsets within the $CD4^+CD27^-$ population of human lymphocytes. *The Journal of Immunology*, 1995, 154: 4294–4301.

T lymphocytes have been subdivided into naive cells and memory cells that respond to recall Ag (1). As lymphocytes develop into memory cells following antigenic stimulation, they cease to express CD45RA and begin to express CD45R0 on their surface (2, 3). The memory $CD4^+$ T cell population has been subdivided further into two functionally distinct subsets on the basis of their cytokine profiles (4, 5). The Th1 cells produce IFN-γ and IL-2 but no IL-4 nor IL-5, whereas Th2 cells produce IL-4 and IL-5 but no IFN-γ nor IL-2. The Th1 cytokines induce important cellular responses that are, for example, central to the elimination of intracellular pathogens (6–9). The Th2 cytokines induce distinct responses as well, most notably the induction of IgE (10, 11) and eosinophilia (12, 13). These Th2 responses are characteristic of helminth infections (14–16), of atopic disorders (17), and of uncontrolled infections with intracellular pathogens (e.g., visceral leishmaniasis (8, 18) and lepromatous leprosy (19)).

The majority of memory ($CD45R0^+$) $CD4^+$ T cells also express the surface marker CD27, a member of the TNF-R/NGF-R tumor necrosis factor receptor/nerve growth factor receptor superfamily (20). On activation of these cells, the density of CD27 on the surface increases; however, on extended stimulation, the molecule is cleaved proteolytically and lost from the cell surface (21, 22). Thus, $CD4^+CD27^-$ cells are considered to be functionally differentiated memory T cells (20).

Previous studies with cells from atopic individuals found that $CD4^+CD27^+$ T cells produce IL-2 but little IL-4, whereas the $CD4^+CD27^-$ cells produce IL-4 but little IL-2 (23). Extension of these studies with use of cells from individuals with helminth infections revealed that not only did these individuals have higher frequencies of

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$CD4^+CD27^-$ lymphocytes (24, 25), but also that purified $CD4^+CD27^-$ lymphocytes produced significantly higher levels of IL-4 and IL-5 than the $CD27^+$ lymphocytes (24); however, the $CD27^-$ cells also produced IFN-γ, suggesting that functionally differentiated $CD4^+$ T cells, both Th1 and Th2, are $CD27^-$ (24).

Before the present study, the definitions of the Th1/Th2 cell dichotomy in humans came from studies of cell culture supernatants of complex mixtures of lymphocytes, frequency analysis by ELISPOT assays, and studies with use of T cell clones (cells that have been manipulated in their generation). To study fresh, ex vivo cytokine production at the single cell level, we have magnetically sorted PBMC and performed flow cytometry for intracellular cytokines. By using these techniques, we have demonstrated differences in the frequencies of T lymphocytes producing various combinations of IL-4, IL-5, and IFN-γ in $CD27^+$ and $CD27^-$ $CD4^+$ cell subpopulations and, in so doing, have been able to define the Th1/Th2 phenomenon more precisely.

Materials and Methods

Study population

The individuals entered into this study following informed consent were returned expatriates (North Americans) with confirmed, active helminth infections ($n = 3$), one individual with a syndrome associated with extreme elevations of circulating eosinophils, and four normal blood bank donors. For all individuals studied, the eosinophil counts ranged from 86 to 4300/ml, and serum IgE levels ranged from 35 to 514 ng/ml.

mAbs

The mAbs used were obtained either commercially (CD4-phycoerythrin and goat anti-mouse IgG conjugated to FITC (Becton Dickinson, San Jose, CA), goat anti-rat IgG conjugated to FITC (Caltag, San Francisco, CA), goat anti-DNP conjugated to FITC (Molecular Probes, Eugene, OR), and streptavidin-phycoerythrin/Cy5 (Life Technologies, Inc., Grand Island, NY)), from ATCC (glycophorin (10F7), CD14 (63D3)), from R&D Systems (Minneapolis, MN) anti-IL-γ (3004-biotin and 3010-biotin or as gifts (IL-4 (3004-biotin and 3010-biotin; R&D Systems, Minneapolis, MN), IL-5 (5A10-biotin; PharMingen, San Diego, CA), IFN-γ (IFGCP-F1BA10, hereafter referred to as BA10; J. Jaffe), CD27 (CLB-CD27/1, R. van Lier CLB, Amsterdam, The Netherlands; 1A4, C. Morimoto, Harvard Medical School, Boston), CD19 (FMC63; H. Zola, Womans & Childrens Hospital, Adelaide, Australia), CD8 (B9.8; B. Malissen, CIML Marseille, France), CD16 (3G8; J. Jaffe, Hahnemann University, Philadelphia), and CD14 (3C10; J. Jaffe)). BA10 was derivatized with DNP (Molecular Probes) according to manufacturer's instructions.

Lymphocyte selections

PBMCs were obtained after apheresis and then were purified further by centrifugation over a Ficoll-diatrizoate (LSM, Organon Teknika Corporation, Durham, NC) gradient. The PBMCs were then purified for $CD4^+$ T lymphocytes by using magnetic beads, as described previously (24). The cells were assessed for purity by flow cytometric analysis. Generally, the purified population was 85 to 95% $CD4^+$.

The $CD4^+$ lymphocytes were then separated into the $CD27^+$ and $CD27^-$ subpopulations by using another magnetic cell-sorting technique (MACS; Miltenyi Biotec Inc., Sunnyvale, CA). The $CD4^+$ cells were incubated at 2×10^7/ml HBSS/0.2% BSA for 30 min at 4°C with 1A4 (1/1000) and CLB-CD27/1 (1/500), washed in ice-cold HBSS/BSA, and incubated for 20 min with goat anti-mouse IgG MACS MicroBeads (Miltenyi Biotec Inc.) at 20 μl beads and 80 μl HBSS/BSA per 10^7 cells. The beads and cells were then passed over a MACS separation column (size C) while on the MACS magnet, and the flow rate was controlled by using a 21G needle. $CD27^-$ cells were washed through with 50 ml HBSS/BSA buffer and collected on ice. The column was removed from the magnet and the $CD27^+$ cells on the beads were flushed out in 100 ml HBSS/BSA. Both subpopulations were washed, counted, and resuspended to 2×10^6/ml in RPMI 1640 containing glutamine (2 mM), HEPES (1 mM), and gentamicin (80 μg/ml) (CRPMI), with 10% FCS (BioWhittaker, Walkersville, MD) for culture. A small aliquot of cells was saved for flow cytometric analysis to assess the purity of the populations.

Table I. *Abs used for intracellular staining of cytokines*

Cytokine	Primary mAb	Secondary mAb
IFN-γ	BA10-DNP 1 μg/ml 3004-Biotin 2.5 μg/ml	Goat anti-DNP-FITC
IL-4	3010-Biotin 2.5 μg/ml	Streptavidin-phycoerythrin/Cy-5[a]
IL-5	5A10-Biotin 1 μg/ml	Streptavidin-phycoerythrin/Cy-5
IL-5	5A10 10 μg/ml	Goat anti-rat IgG-FITC[a]

[a] Combination used for Figure 5.

Cell cultures

The purified lymphocyte subpopulations were cultured for 6 h (previously found to be the optimal time for detection of this combination of cytokines) at 37°C and 5% CO_2 and stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin (Calbiochem, La Jolla, CA) in the presence of 2 μM monensin (Calbiochem). Monensin inhibits cytokine secretion, resulting in cytokine accumulation within the cells (26). The cells were then treated with 20 μg/ml DNase for 5 min at 37°C, harvested, washed twice with ice-cold PBS, and fixed with 4% paraformaldehyde at 37°C for 5 min. The cells were then washed with ice-cold PBS/1% BSA and cryopreserved at −70°C in PBS/1% BSA/10% DMSO.

Three-color flow cytometry for intracellular cytokines

Cryopreserved cells were thawed at 37°C, washed in PBS/0.1% BSA, and then incubated overnight on ice at 4×10^6/ml in PBS-S[3] (PBS, 0.01 M HEPES, 0.1% saponin (Fluka Chemicals, Ronkonkoma, NY), 0.1% BSA, pH 7.4) with 10 μg/ml of streptavidin (Life Technologies, Inc.), 10% dialyzed human serum, and 2% mouse serum to block endogenous biotin and nonspecific binding, respectively. The cells were then washed twice in PBS-S and incubated at 4×10^6/ml in PBS-S/10% human serum with 2 μM biotin (Pierce Chemicals, Rockford, IL) for 30 min on ice. After aliquoting into 4-ml conical bottom tubes (Sarstedt, Newton, NC), the cells (200,000/tube) were incubated for 30 min on ice with the anti-cytokine mAbs, as specified in Table I. After washing twice with PBS-S, they were resuspended in 50 μl PBS-S/10% human serum and incubated with the secondary Abs (as described in Table I) for 30 min on ice. The cells were then washed twice in PBS-S and finally resuspended in 300 μl PBS.

To ensure the specificity of the staining procedure, the binding of each Ab was blocked with a molar excess of recombinant cytokine (IFN-γ, Immunex, Seattle, WA; IL-4, Immunex; and IL-5, Genzyme Corp., Cambridge, MA). Routinely, each sample had a control in which specific binding was blocked by using either a molar excess of unlabeled mAb (IFN-γ and IL-5) or recombinant cytokine (IL-4). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson). The typical forward and side scatter gate for lymphocytes together with a $CD4^+$ gate were set to exclude any dead and contaminating, non-$CD4^+$ lymphocytes from the analysis; 30,000 events within this gate were acquired per sample. Two-parameter histograms demonstrating cytokine staining were created by using LYSIS II software (Becton Dickinson), and quadrant statistics were placed on the basis of the staining of specifically blocked negative controls.

To compare this new technique with the existing technique of measuring secreted cytokines in cell supernatants, the $CD4^+$ lymphocytes of

[3] Abbreviations used in this paper: PBS-S, phosphate buffered saline-saponin (0.1%); GM, geometric mean.

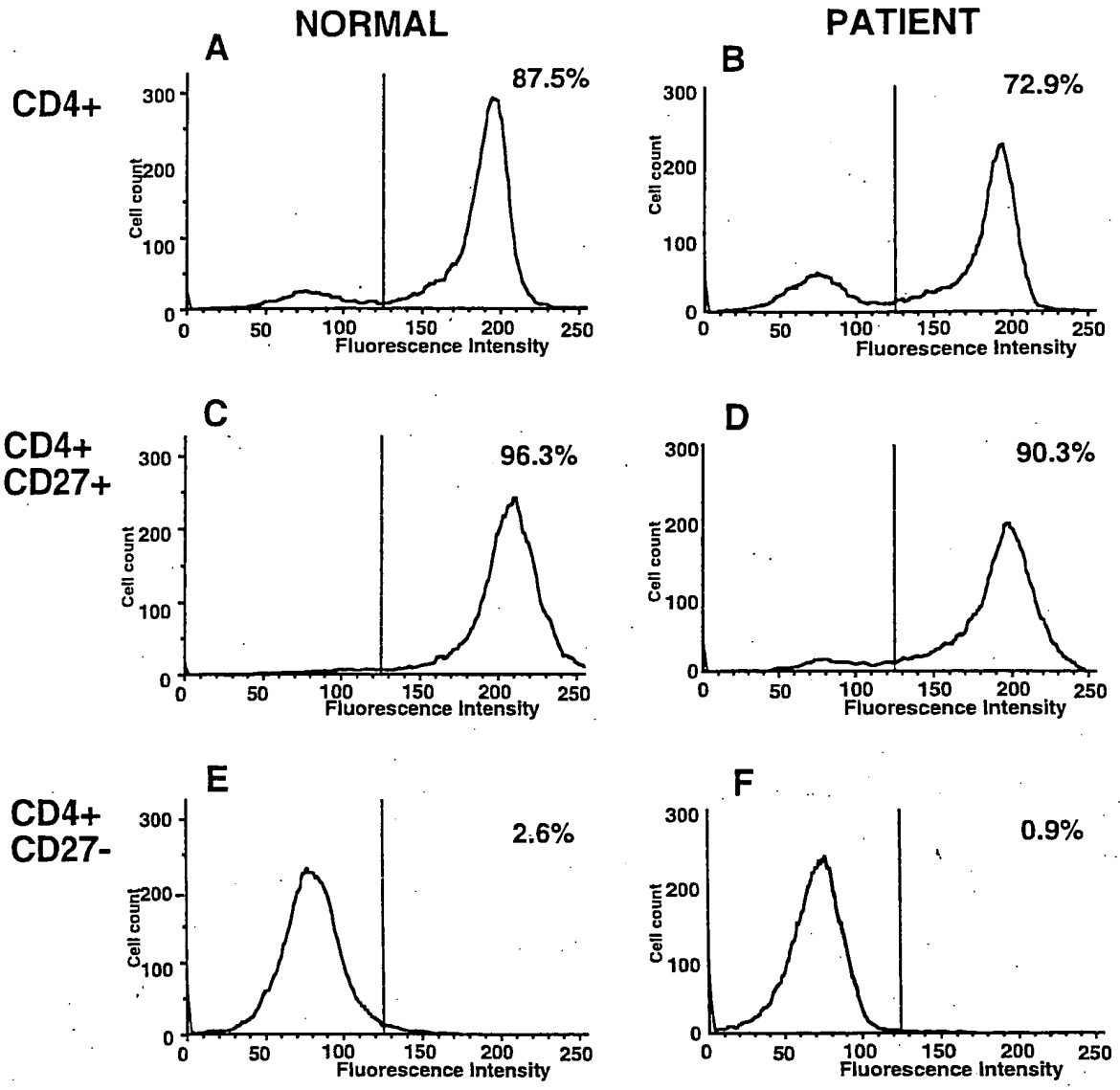

FIGURE 1. CD27 expression of magnetically sorted lymphocyte subpopulations. One-parameter histograms of CD27 expression are shown for the negatively selected $CD4^+$ population (*A* and *B*) as a whole, before being further selected by their expression of CD27, and for the two subpopulations resulting from positive selection, the $CD4^+CD27^+$ population (*C* and *D*), and from negative selection, the $CD4^+CD27^-$ population (*E* and *F*). Histograms for one helminth-infected patient (*B*, *D*, and *F*) and one normal individual (*A*, *C*, and *E*) are shown. The *vertical line* marks the cutoff used for assessing frequencies of positive cells as defined by the binding of the control Ab.

five patients and one normal individual were magnetically purified and cultured in vitro. The lymphocytes were divided into two and cultured either in the presence of PMA, ionomycin, and monensin for 6 h for intracellular staining of cytokines, or in the presence or absence of PMA and ionomycin for 48 h for supernatant measurement of IL-4, IL-5, and IFN-γ, as described previously (27). The frequencies of cytokine-producing cells were then compared with the amounts of each cytokine secreted into the supernatant by using simple linear regression analysis.

Statistical analysis

Statistically significant differences were assessed by using the Mann Whitney-*U* test or the Wilcoxon signed rank test, and correlations were performed by using simple linear regression analysis.

Results

Purity of selected CD27 subpopulations

The magnetically sorted CD27 subpopulations were checked for purity by flow cytometry by using an anti-CD27 mAb (CLB-CD27/1) followed by goat anti-mouse FITC. Figure 1 shows the CD27 expression of the unsorted $CD4^+$ population and the sorted CD27 subpopulations from a representative normal donor (Fig. 1, *A*, *C*, and *E*) and from a helminth-infected patient (Fig. 1, *B*, *D*, and *F*). The $CD27^+$ fraction of cells ranged from 87 to 98% $CD27^+$ (Fig. 1, *C* and *D*), whereas the $CD27^-$ fraction

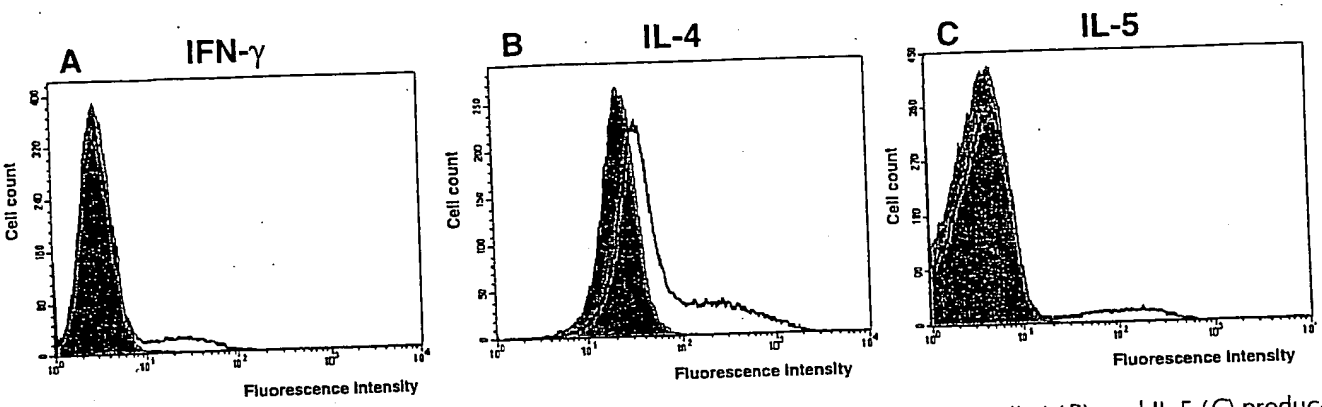

FIGURE 2. Intracellular cytokine staining is specific. One-parameter histograms of IFN-γ (*A*), IL-4 (*B*), and IL-5 (*C*) production are shown for the $CD4^+$ lymphocytes of one patient. The histograms illustrate the intracellular staining obtained in the presence (shaded) or absence (unshaded, bold line) of a molar excess of specific recombinant cytokine.

ranged from 93 to 99% $CD27^-$ (Fig. 1, *E* and *F*) for all individuals in the study.

Specificity of intracellular cytokine staining

Magnetically sorted $CD4^+$ lymphocytes from one patient were stimulated, in vitro, for 6 h with PMA and ionomycin in the presence of monensin, and were then fixed with paraformaldehyde. The lymphocytes were permeabilized with saponin and stained for CD4 and either IFN-γ (Fig. 2*A*), IL-4 (Fig. 2*B*), or IL-5 (Fig. 2*C*) in the presence (*shaded*) or absence (*unshaded, bold line*) of a molar excess of the corresponding recombinant cytokine and were analyzed by flow cytometry. Figure 2 illustrates the one-parameter histograms for the cytokine expression of the gated $CD4^+$ lymphocytes. The anti-cytokine staining revealed a distinct proportion of the $CD4^+$ lymphocytes expressing each cytokine. Addition of the recombinant cytokines clearly blocked the binding of the Abs to the cells, demonstrating the specificity of the staining.

Correlation of intracellular cytokine staining with levels of secreted cytokine

To compare the flow cytometric technique with the existing technique of measuring cytokines secreted into cell supernatants, the $CD4^+$ lymphocytes of five patients and one normal individual were magnetically purified, cultured in vitro in the presence or absence of PMA, ionomycin, and/or monensin, and the frequencies and cytokine levels in supernatant were assessed in parallel. Linear regression analysis revealed that the frequencies of $CD4^+$ lymphocytes expressing IL-4 and IL-5 were correlated directly with the amounts of IL-4 and IL-5 secreted into the culture supernatants ($r^2 = 0.918$, $p = 0.003$ and $r^2 = 0.787$, $p = 0.018$, respectively). Analysis of the frequency of $CD4^+$ lymphocytes expressing IFN-γ, however, revealed that there was little correlation with the amount of IFN-γ measured in the culture supernatants ($r^2 = 0.339$, $p = 0.225$; data not shown).

Production of IL-5 and IFN-γ is mutually exclusive

The purified $CD4^+CD27^+$ and $CD4^+CD27^-$ T lymphocyte subpopulations from four patients and four normal individuals were stained fluorescently for IL-5, IFN-γ, and CD4, or IL-4, IFN-γ, and CD4, and analyzed by flow cytometry. The two-parameter histograms for one representative patient are illustrated in Figure 3. Remarkably, virtually no lymphocytes had the capacity to produce both IL-5 and IFN-γ (Fig. 3, *A* and *C*), whereas a distinct subset of both the $CD27^-$ and $CD27^+$ lymphocytes was able to produce both IL-4 and IFN-γ (Fig. 3, *E* and *G*). The two CD27 subpopulations, however, very clearly differed in their frequencies of cells producing IL-4, IL-5, and/or IFN-γ. The $CD4^+CD27^-$ subpopulation had a higher frequency of cells capable of producing any of the three cytokines examined than the $CD4^+CD27^+$ population. Within the $CD4^+CD27^-$ population, there was a higher frequency of cells capable of producing IL-4 (25%) or IFN-γ (26%) than IL-5 (7%).

Most cytokine-producing $CD4^+$ lymphocytes of both patients and normal individuals are $CD27^-$

To determine whether the segregation of cytokine-producing cells into distinct functional subsets was a general phenomenon, cells from four patients and four normal individuals were analyzed in the same manner. These analyses clearly show that most individuals possess only a low frequency of cells co-expressing IL-5 and IFN-γ (Fig. 4*B*), whereas most individuals had a distinct subset co-expressing IL-4 and IFN-γ (Fig. 4*E*). All individuals had higher frequencies of cells producing either IL-4, IL-5, or IFN-γ alone than cells co-expressing IL-5 and IFN-γ or IL-4 and IFN-γ.

The previously noted differences between the two CD27 subpopulations were also seen to hold for both patients and normal individuals. The $CD4^+CD27^-$ population consisted of significantly higher frequencies of cells that were $IL\text{-}5^+IFN\text{-}\gamma^-$ (GM, 4.9% vs 1.5%;

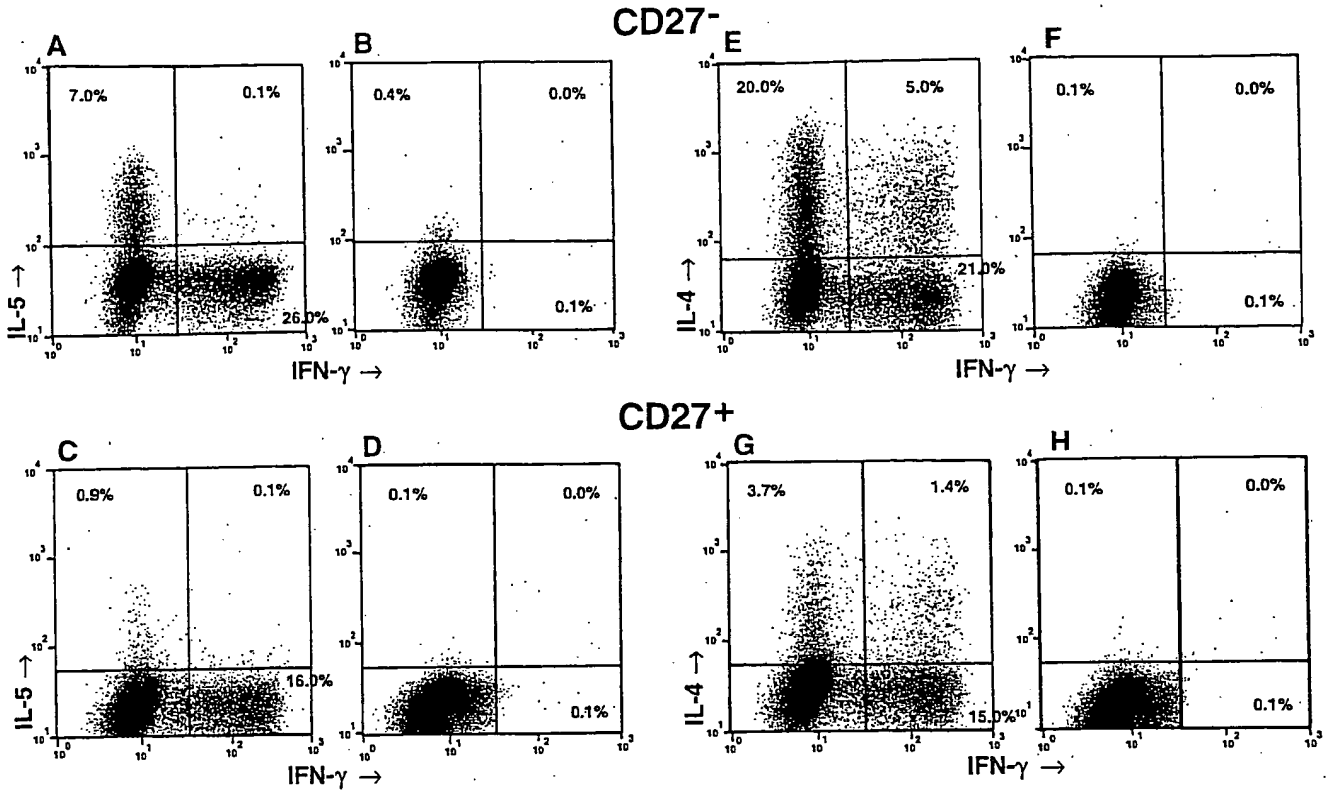

FIGURE 3. $CD4^+$ lymphocytes rarely produce both IL-5 and IFN-γ, but a distinct proportion produce both IL-4 and IFN-γ. Two-parameter dot plots of IL-5 and IFN-γ production (*A–D*) and of IL-4 and IFN-γ production (*E–H*) are shown for the $CD4^+CD27^-$ (*A, B, E,* and *F*) and the $CD4^+CD27^+$ (*C, D, G,* and *H*) lymphocyte subpopulations from one helminth-infected patient. Quadrant statistics were set on the basis of the corresponding blocked, negative controls (*B, D, F,* and *H*).

$p = 0.025$; Fig. 4*A*), $IL\text{-}4^+IFN\text{-}\gamma^-$ (GM, 13.8% vs 3.5%; $p = 0.025$; Fig. 4*D*), and $IFN\text{-}\gamma^+IL\text{-}4^-IL\text{-}5^-$ (GM, 27.3% vs 12.0%; $p = 0.012$; Fig. 4, *C* and *F*) than the $CD4^+CD27^+$ population. The $CD4^+CD27^-$ population also contained a significantly higher frequency of cells capable of producing both IL-4 and IFN-γ than the $CD27^+$ population (GM, 2.4% vs 1.0%; $p = 0.017$; Fig. 4*E*).

Patients and normal individuals only differed in the frequency of $CD4^+CD27^-$ cells capable of producing a given cytokine. The normal individuals had significantly higher frequencies of cells producing IFN-γ (GM, 44.5% vs 17.2%; $p = 0.043$), whereas the patients showed a trend toward higher frequencies of cells producing IL-4 (GM, 24.3% vs 16.4%) and/or IL-5 (GM, 10.2% vs 2.9%). Linear regression analysis revealed that the frequency of $CD4^+CD27^-$ cells capable of producing IFN-γ is inversely proportional to the frequency of cells capable of producing IL-5 ($p = 0.002$; $r^2 = 0.831$).

IL-4 and IL-5 production

To determine what proportion of the IL-5-producing cells also produce IL-4, magnetically sorted lymphocyte populations were stained for IL-4, IL-5, and CD4. By using PBMCs from a helminth-infected patient (Fig. 5), it can be seen that 75% of the IL-5-producing $CD4^+CD27^-$ lymphocytes also produced IL-4. This was seen to be true for all patients and normal individuals analyzed (66–84%; data not shown). This $IL\text{-}5^+IL\text{-}4^+$ population represented 3 to 54% of the IL-4-producing $CD4^+CD27^-$ lymphocytes.

Discussion

We have used intracellular staining for cytokines, combined with flow cytometry, to examine the frequencies of cytokine-producing cells in the $CD4^+CD27$ T lymphocyte subsets from both patients and normal individuals. A unique strength of this technique is the ability to assess multiple cytokines simultaneously at the single cell level. Direct comparison with the existing technique of measuring levels of cytokines secreted by lymphocytes into culture supernatants demonstrated that this is directly comparable to the frequency of cells obtained from intracellular staining for IL-4 and IL-5, a finding that has also been demonstrated previously by using human T cell clones (28). These findings indicate that an increase in IL-4 and IL-5 production is solely caused by an increase in the number of cells secreting the cytokine. The poorer correlation seen for IFN-γ production suggests that an increase in the levels of IFN-γ measured in

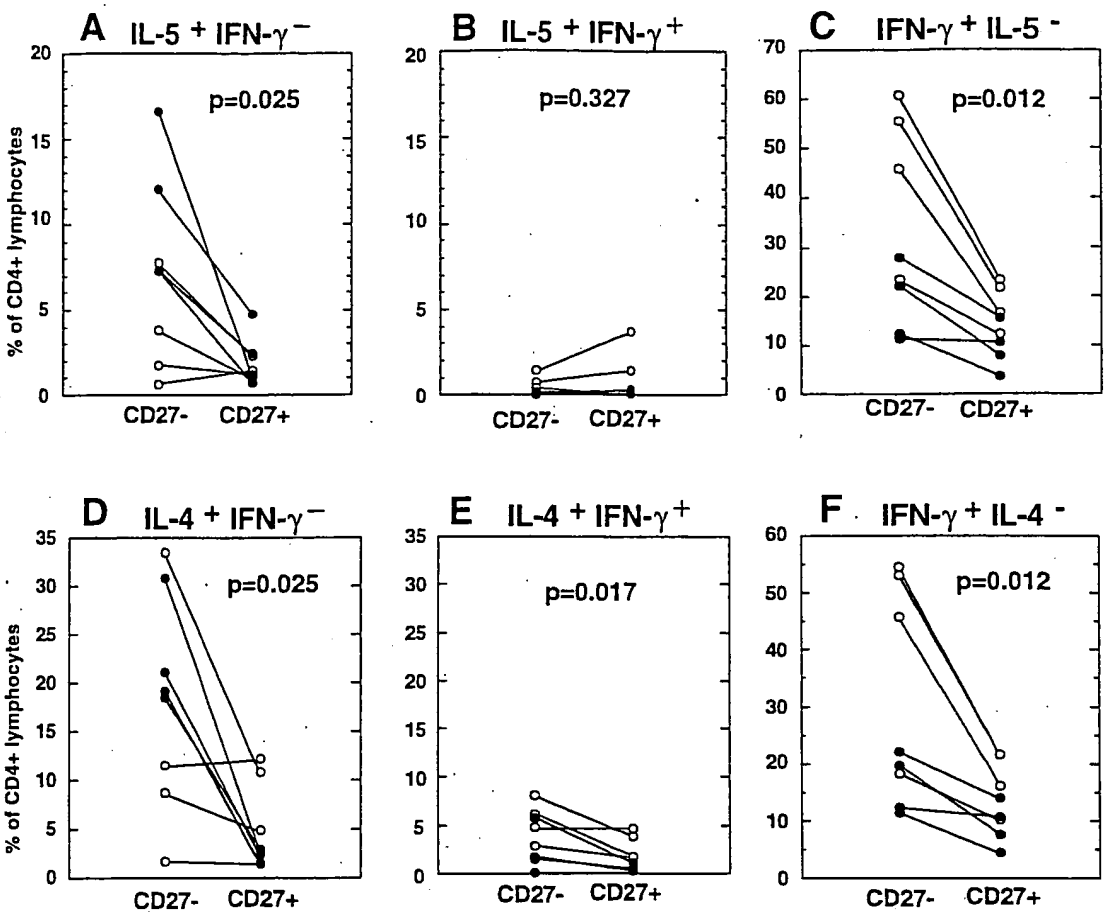

FIGURE 4. The $CD4^+CD27^-$ lymphocyte subpopulation contains significantly higher frequencies of cells producing IL-4, IL-5, and/or IFN-γ. The percentages of $CD4^+$ lymphocytes producing each possible combination of IL-5 and IFN-γ (*A–C*), and IL-4 and IFN-γ (*D–F*) are shown for the $CD27^+$ and $CD27^-$ subpopulations from helminth-infected patients (●) and normal individuals (○). The two dots joined by a line represent the values for the two cell subpopulations from the same individual. *p* values indicate levels of significant differences between the CD27 subpopulations with use of the Wilcoxon signed rank test. Note: Because the frequencies of cells producing IFN-γ were higher than those for IL-4 and IL-5, the scales for the IFN-γ graphs (*C* and *F*) are different from those for IL-5 and IL-4 (*A, B, D,* and *E*).

supernatants may be caused not only by an increase in the numbers of IFN-γ-producing cells, but also in the amount of IFN-γ released by individual cells.

This new technique has enabled us to demonstrate that $CD4^+$ T lymphocytes from both patients and normal individuals have only a very low frequency of cells producing a combination of IL-5 and IFN-γ, a distinct subset producing IL-4 and IFN-γ, but have higher frequencies of cells producing IL-4, IL-5, or IFN-γ alone. This phenomenon clearly echoes some of the Th1/Th2 paradigm, but differs to some degree, as well. Previously, Th2 cells have been defined as producing IL-5 and IL-4. In this work, we have shown that of the $CD4^+CD27^-$ cells that produce IL-5, 66 to 84% also produce IL-4, and that these represent 3 to 54% of the IL-4-producing cells. Clearly, there are distinct numbers of cells that produce only IL-5 or only IL-4. This agrees with recent data using in situ hybridization that demonstrated that even within a cloned Th2 murine lymphocyte population, there was a higher frequency of cells expressing IL-4 mRNA than IL-5 mRNA, and thus, a distinct subpopulation expressing only IL-4 mRNA (29). At present, we are not able to assess what proportion of these cells is also able to produce IL-2 and, thus, we cannot conclusively define them as Th2 cells. Nevertheless, it appears that the Th2 definition may need to be redefined as being the production of IL-4 and/or IL-5, not necessarily both.

Another subset of lymphocytes that produce IL-4 has previously been designated Th0 cells. These were generally thought to be the common precursor of Th1 and Th2 cells, as they produce a combination of Th1 and Th2 cytokines and appear transiently after activation of naive T cells (1, 30, 31). The present study, however, has demonstrated that although 0.1 to 8.0% of the $CD4^+CD27^-$ population produce both IL-4 and IFN-γ, which confirms a previous study using fluorescent microscopy (32), only 0.03 to 1.4% produce both IL-5 and IFN-γ, indicating that Th0 cells produce IL-4 and IFN-γ, but very rarely IL-5.

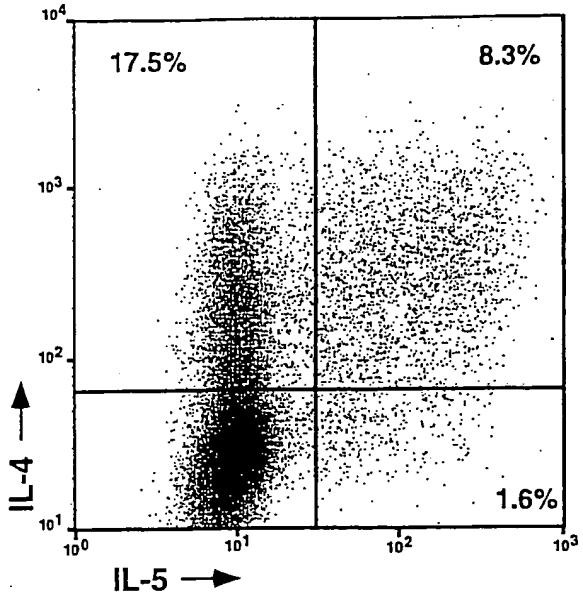

FIGURE 5. Most IL-5-producing cells also produce IL-4. A two-parameter dot plot of IL-5 and IL-4 production is shown for the $CD4^+CD27^-$ lymphocyte subpopulation from one helminth-infected patient.

The ability to stain intracellularly for cytokines has also allowed for the identification of functional differences between the $CD4^+CD27^+$ and $CD4^+CD27^-$ T cell subpopulations. The frequencies of all cytokine-producing cells were significantly higher in the $CD4^+CD27^-$ subset of both patients and normal individuals, confirming previous studies that CD27 expression does not differentiate Th1 and Th2 lymphocytes, but that all functionally differentiated T cells are $CD27^-$ (20, 24); however, the cells that co-expressed IL-4 and IFN-γ, the Th0-like cells, were also seen at a higher frequency in the $CD4^+CD27^-$ population than in the $CD4^+CD27^+$ population. This suggests either that Th0 cells are as equally differentiated as the Th1 and Th2 cells or that they are a transitory precursor stage subsequent to prolonged Ag exposure, as suggested by previous studies (1, 30). Those studies have shown that stimulation of naive murine $CD4^+$ T cells via the Ag receptor induced only IL-2 production, but on prolonged stimulation, these cells produced more IL-4 and IFN-γ than IL-2 and then differentiated into cells producing only IL-4 or only IFN-γ, depending on the nature of the cytokine environment at the time of differentiation (1, 30).

Analysis of patients and normal individuals demonstrated that the functional T cell subsets are ubiquitous. The patients tended to have higher frequencies of $CD4^+$ $CD27^-$ cells producing IL-5 and/or IL-4, but lower frequencies capable of producing IFN-γ than the normal individuals. Indeed, a statistically significant, negative correlation was seen between the frequency of cells capable of producing IL-5 or IFN-γ for the study population as a whole ($r^2 = 0.831$; $p = 0.002$). This indicates not only that the cells that produce IL-5 and IFN-γ are very distinct, but also that when exposed to a pathogen that can induce a Th2-type cytokine profile, the differentiated T cell population ($CD4^+CD27^-$) is dominated by the Th2 lymphocytes. This suggests that helminth-infected patients may not be able to mount an effective Th1-type memory response, as would be required for protection against bacterial, viral, or intracellular protozoan infection.

Although the time period chosen for stimulation of the cells was found to be optimal for this mitogenic stimulation, it is important to remember that the data represent a fixed time point in the development of the cytokine responses of these cells after in vitro stimulation with mitogen. Intracellular staining for cytokines coupled with flow cytometry should enable the study of the development of cytokine production by individual T cells as they move from their naive state, through their first encounters with specific Ag, to the development of antigen-specific functional subsets such as the Th1 and Th2 cells. Thus, we have shown, virtually ex vivo, an exclusivity in the production of IL-5 and IFN-γ, a distinct coproduction of IL-4 and IFN-γ, and extensive coproduction of IL-4 and IL-5, demonstrating the presence of Th1, Th2, and Th0 cell subsets within the $CD4^+CD27^-$ subpopulation of human PBMCs.

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Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies

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Abstract

Recently, there have been several reports demonstrating improvements in the flow cytometric detection of intracellular cytokines. These advances, although significant, have not yielded techniques that have easily been translated into broad use. To address this issue, we have coupled a fixation and permeabilization method with the use of directly labelled monoclonal anti-cytokine antibodies, providing both improved signal and simpler staining. The kinetics of in situ cytokine production in both CD4 and CD8 cells are shown for IL-2, IL-4, IL-5 and IFN-γ. Based on these data, 6 h was chosen for optimal detection of this combination of cytokines. We show the specificity of this technique by blocking cytokine staining using a molar excess of recombinant cytokine. Additionally, unlabelled anti-cytokine antibodies are demonstrated to block specific staining of labelled antibody, providing an objective means to place statistical markers. Using such controls, we routinely detected as few as 0.1% false positive cells, allowing the flow cytometric detection of IL-5, which is below the threshold of detection of published methods. To further prove the specificity of staining, we stained using two anti-IL-5 mAbs known to recognize different epitopes and demonstrate that the same cells stain with both antibodies. Without permeabilization we could detect a fraction of cells with low intensity staining for cytokine. This staining was further examined using differential two color staining for intracellular and extracellular cytokine, clearly demonstrating no cells staining exclusively for extracellular cytokine, confirming a lack of passive transfer of cytokine to nearby cells. We show that cytokine flow cytometry is useful in examining the increased IL-5 production characteristic of eosinophilic states and that IL-5 production is limited to the CD27 negative subpopulation. These data illustrate the unique capability of cytokine flow cytometry to correlate cytokine expression with cell surface phenotype without cell separation. In summary, using directly conjugated anti-cytokine antibodies, cytokine flow cytometry becomes a specific and versatile technique for the assessment of complex cytokine production phenotypes in fresh ex vivo T cell subpopulations.

Keywords: Flow cytometry; Cytokine; Monensin; Intracellular immunofluorescence; T cell; Th_1 cell; Th_2 cell; IL-5

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DNP, dinitrophenyl; FITC, fluorescein isothiocyanate; FSX, fluorescein-X-succinimidyl ester; IL, interleukin; IFN, interferon; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PE, phycoerythrin; PFA, paraformaldehyde; PMA, phorbol 12-myristate acetate.

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1. Introduction

Lymphocytes as well as other cells of the immune system exert their effector function in part through the synthesis and release of cytokines. The production of cytokines by such cells may be studied on both the protein and mRNA level, typically by measuring the bulk production of cytokine protein or message. However, it is clear that the cytokine production of a cell population may not reflect expression at the single cell level, and that a number of significant immunologic questions require the analysis of cytokine production by individual cells (Umlauf et al., 1993; Bucy et al., 1994; Cassell and Schwartz, 1994). One such question results from the recognition of a dichotomy in T helper cytokine production separating T cells into Th_1 and Th_2 subsets. This paradigm is based primarily on results obtained from T cell clones, which are long term in vitro propagated lines. Studies in fresh ex vivo T cells, by immunofluorescence microscopy, similarly reveal differences in cytokine patterns (Andersson et al., 1990).

Attempts to further extend these observations in ex vivo T cells require a single cell cytokine assay that measures two or more cytokines simultaneously. Single cell assays employed in this manner include ELISPOT, limiting dilution analysis and in situ hybridization, all of which are labor intensive or technically demanding. The multiparameter capability of flow cytometry has also been adapted to measurement of two cytokines simultaneously by single cells and has the advantage of rapidly determining the cytokine production of a large number of individual cells. Accordingly, several groups have reported the flow cytometric detection of intracellular cytokines to explore such areas (Andersson et al., 1988; Jung et al., 1993; Assenmacher et al., 1994). These studies have employed unlabelled anti-cytokine antibodies which require elaborate staining techniques and severely limit the choice of antibodies that can be used.

To improve the sensitivity of the flow cytometric detection of cytokine production, we have modified a method first reported by Sander and Andersson (1991) and in conjunction with the use of directly conjugated anti-cytokine monoclonal antibodies we have significantly lowered nonspecific binding, thus increasing the signal to noise ratio and sensitivity of the assay. These modifications now allow the routine detection and analysis of cytokines including IL-5, which heretofore has been below the threshold of detection of previously described flow techniques. The use of directly conjugated anti-cytokine antibodies also enables us to block specific binding with unlabelled antibody prior to staining, allowing for a more rigorous definition of positive and negative staining cells. Further, the staining procedure itself is greatly simplified with these directly labelled reagents. These modifications thus allow for the analysis of single cell cytokine production at a level of detection which was previously not possible and which may provide substantial new insight into cytokine production at the single cell level.

2. Materials and methods

2.1. Antibodies

Anti-CD3 fluorescein isothiocyanate (FITC) and anti-CD8 phycoerythrin (PE)/Cy5 (Pharmingen, San Diego, CA); anti-CD4 FITC (Becton-Dickinson, San Jose, CA); anti-CD4 PE/Cy5 (Sigma Chemical Co., St. Louis, MO); anti-CD27 (Research Diagnostics, Flanders, NJ) and goat anti-mouse IgG PE/Cy5 (Caltag, South San Francisco, CA) were obtained from the manufacturers. The following anti-cytokine antibodies were obtained as custom conjugates or conjugated in our laboratory and may not be commercially available: anti-IL-2: mAb 5322.111 was obtained from R&D systems (Minneapolis, MN) and was fluorescein-X-succinimidyl ester (FSX) and PE labelled; anti-IFN-γ, clone IFGCP-F1BA10 (BA10) was obtained from American Type Culture Collection (Rockville, MD) and FSX and PE labelled; anti-IL-4 mAbs 25D2 and 8D4 (PE and FITC) and anti-IL-5 mAbs 5A10 and 39D10 (PE and FITC) were gifts from Pharmingen. MAbs 5322, 25D2, 8D4, 39D10 and 5A10 have all been described by to be neutralizing antibodies by their respective suppliers.

2.2. Reagents

Ionomycin and monensin (Calbiochem, La Jolla, CA); saponin, phorbol 12-myristate acetate (PMA) and DNAse I (2000 U/mg) (Fluka, Ronkonkoma,

NY); FSX (Molecular Probes, Eugene, OR); and paraformaldehyde (PFA) and dimethyl sulfoxide (DMSO) (Sigma) were obtained commercially.

2.3. Cell culture

Peripheral blood mononuclear cells (PBMC) were either obtained from buffy coats of normal donors acquired from the NIH Clinical Center Blood Bank, or following informed consent from patients with elevated eosinophil counts associated with parasitic diseases. PBMC were separated by Ficoll-diatrizoate (LSM, Organon-Technica; Durham, NC) density gradient separation. Cells were suspended in RPMI supplemented with 10% fetal calf serum (Biofluids, Rockville, MD), 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25 μg/ml gentamicin, 50 μM 2-mercaptoethanol, 1 mM pyruvate and nonessential amino acids (Biofluids) at a density of 2×10^6 cells/ml. Cells were then stimulated in 6 well plates in a volume of 10 ml with 1 μM ionomycin and 20 ng/ml PMA. Monensin (2 μM) was also added at the start of culture to inhibit cytokine secretion (Tartakoff, 1983; Henter et al., 1988; Lee et al., 1990). At harvest, 40 μg/ml DNAse was added to each well to decrease cell aggregation. Cells were removed from the plate with a cell scraper at specified times, the contents of each well placed in a 15 ml V bottom tube, and incubated at 37° for 5 min to allow disaggregation of cells. The cells were then centrifuged at $500 \times g$ for 10 min and washed once in ice cold phosphate buffered saline (PBS). 3 ml of 4% PFA in 0.1 M phosphate buffer (pH 7.4) warmed to 37°C were added to the cell pellet and intermittently pipetted up and down for 5 min. 12 ml of ice-cold PBS with 0.1% bovine serum albumin (BSA) (PBS-BSA) were added to each tube to stop fixation, the tube capped, inverted to mix and centrifuged at $1500 \times g$ for 10 min. After aspiration, the resulting cell pellet was resuspended in 1 ml of PBS with 10% dimethyl sulfoxide (DMSO), aliquotted into cryovials and stored at −80°C until analysis.

2.4. Staining for intracellular cytokines

Aliquots of the stimulated and fixed cells were thawed in a 37°C water bath and washed in PBS-BSA. The resultant cell pellet was resuspended in 50 μl PBS with 0.1% saponin, 0.1% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+} (PBS-S) with 5% nonfat dry milk per 10^6 cells for 1 h to block nonspecific binding. The cells were then aliquoted for staining at 200 000 cells per tube into 4.5 ml V bottom tubes (Sardstedt, Germany) and centrifuged at $1500 \times g$ for 10 min. The supernatant was aspirated and the cell pellet resuspended in PBS-S-5% milk containing unlabelled anti-cytokine mAb or isotype control at 100 μg/ml in a total volume of 50 μl for 60 min. Directly labelled anti-cytokine monoclonal antibody (0.3–2.5 μg/ml) was then added to both blocked and stained samples at an optimal concentration. Antibodies to both IL-4 and IL-5 were used as pairs of mAb that recognize different epitopes of the cytokine molecule (Abrams et al., 1992). Antibodies to cell surface markers, when appropriate, were added at the time of addition of labelled anti-cytokine antibodies. The samples were then incubated at 4°C for 30 min, washed twice in PBS-S and resuspended in PBS-BSA for analysis. Recombinant cytokine blocking experiments were performed as above with the addition of a 100–1000-fold molar excess of recombinant cytokine or an equivalent volume of PBS-BSA to the antibody for 1 h prior to addition to the sample.

To differentially stain extracellular (saponin independent) and intracellular (saponin dependent) cytokine, cryopreserved cells were first blocked for 1 h in PBS-BSA-5% milk and aliquotted as above. PE labelled anti-cytokine mAb in PBS-BSA-5% milk was then added. After 30 min, 100 μg of unlabelled anti-cytokine mAb was added for an additional 30 min. The cells were washed twice with PBS-BSA and resuspended in PBS-S-5% milk with the same FITC labelled anti-cytokine mAb and anti-CD4 PE/Cy5. The samples were then incubated for 30 min, washed twice in PBS-S and resuspended in PBS-BSA for analysis.

When used with conventional fluorescent microscopy, direct conjugates require extended exposure times relative to indirect staining. These longer exposure times cause photobleaching which limits detection of positives, making correlation of flow results with microscopy difficult. When used in flow cytometry, where photobleaching is not a problem, these mAbs exhibit a superior signal to noise ratio relative to indirect staining.

2.5. Acquisition and analysis

List mode data was acquired on a FACSCAN flow cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, CA) using LYSIS II software. Dead cells and monocytes were excluded by forward and side scatter gating. Typically, 20000–100000 events were acquired, depending on the cytokine being studied (Parks et al., 1989). List mode files were then analyzed using either LYSIS II or CELLQUEST software. Statistical markers were set using the unlabelled mAb blocked negative controls as a reference. Typically, 0.1% or fewer positive cells were allowed beyond the statistical marker in these controls.

3. Results

3.1. Kinetics of cytokine production

Assays of cytokine production often measure cytokines secreted into the tissue culture supernatant. Presuming negligible consumption, this represents the summation of cytokine synthesis over the incubation period. Examination of cytokine production in situ by flow cytometry yields results for a specific time point and for individual cells, without such integration. It is thus important to select a time for analysis when using flow cytometry that maximizes detection of the cytokines in question. To determine the kinetics of cytokine production as assessed by cytokine flow cytometry, cells from seven normal donors were stimulated with ionomycin and PMA for 4, 6, 10, 12 and 21 h, and fixed with PFA. The cells were then stained with anti-CD4 FITC, anti-CD8 PE/Cy5 and PE labelled antibodies directed against IL-2, IL-4, IL-5 or IFN-γ and analyzed by flow cytometry. Frequencies of cytokine producing cells were then enumerated after gating on either CD4 or CD8 positive cells. IL-2 and IFN-γ were found to peak at 10–12 h (Fig. 1A, E, B and F). In contrast, IL-4 and IL-5 expression was found to peak at 6 h (Fig. 1C, G, D and H). In a subsequent experiment there was a marked decrease in the frequencies of cells producing all cytokines at 24 and 36 h (data not shown). The frequencies of cells producing each cytokine were relatively constant over the 4–12 h time points. These relatively constant determinations over several hours may represent the inhibition of cytokine secretion by monensin, thus maintaining intracellular cytokine levels despite the decrease in de novo synthesis at the latter time points. In succeeding experiments, 6 h was chosen as the incubation time, as it was optimal for IL-4 and IL-5 analysis, and IL-2 and IFN-γ frequencies were routinely 80% of maximal. IL-2 was produced by a greater fraction of CD4 than CD8 cells for all subjects (geometric mean (GM): 66% vs. 30%), whereas the converse was true for IFN-γ (GM: 18% vs. 45%). Significant numbers of IL-4 and IL-5 staining cells were found in both the CD4 and CD8 subsets.

3.2. Blocking of specific staining with recombinant cytokine and unlabelled antibody

Previous studies using cytokine flow cytometry have used unlabelled anti-cytokine monoclonal antibodies followed by addition of labelled secondary antibody. To discriminate positive from negative staining cells, these studies used either isotype matched controls (Jung et al., 1993) or secondary antibody only controls (Assenmacher et al., 1994). However, in our hands, neither of these techniques reproducibly discriminate specific from nonspecific staining. This appears to be because fixation drastically increases nonspecific binding of such antibodies to cells (Larsson, 1993), even with the addition of bland proteins such as serum or milk. Because of this, isotype matched controls may not parallel the nonspecific binding of anti-cytokine antibodies. Secondary antibody only controls in this situation do not account for the nonspecific staining of the primary anti-cytokine antibody and as such may overestimate the number of specifically staining cells.

We have thus employed two approaches to more rigorously discriminate specific staining for cytokine from background or nonspecific staining. First, we have added a molar excess of recombinant cytokine to the anti-cytokine antibody prior to staining to abrogate specific staining (Fig. 2). No discernable alteration in the mean fluorescence of the negative peak was observed. Binding of all of the monoclonal antibodies used in this study has been similarly abrogated using the corresponding recombinant cytokines, typically at a concentration of 10–100

μg/ml. These results thus demonstrate that the immunofluorescence observed is specific to the cytokine in question. Second, our use of directly labelled antibodies has allowed us to employ a more versatile and cost-effective method to assess nonspecific binding. With this technique the negative con-

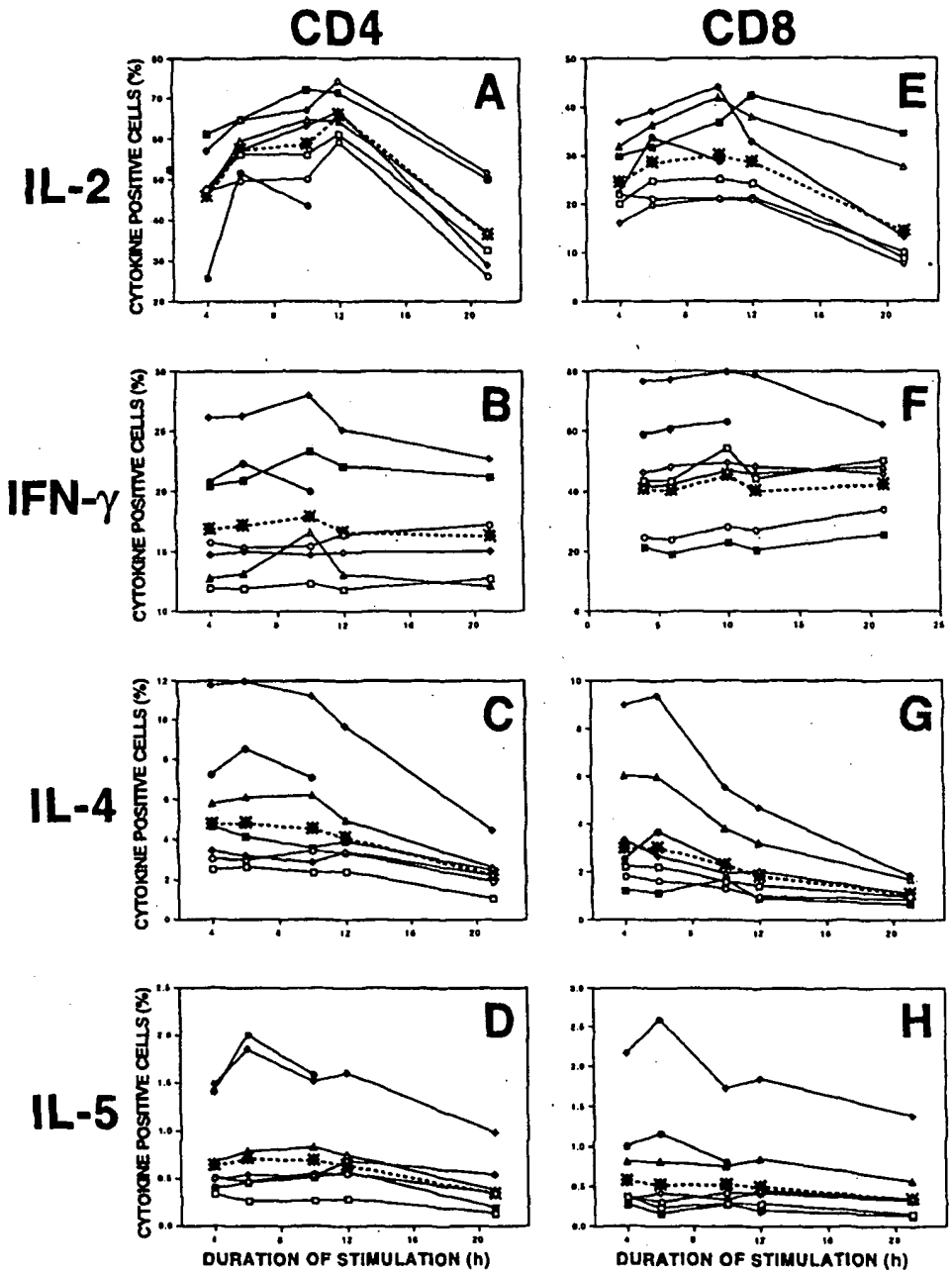

Fig. 1. Time course of cytokine staining. PBMC from seven blood bank donors were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for the indicated times, fixed and stained with anti-CD4 FITC, anti-cytokine PE and anti-CD8 PE/Cy5. Frequencies of IL-2 (*A*, *E*); IFN-γ (*B*, *F*); IL-4 (*C*, *G*) and IL-5 (*D*, *H*) staining CD4 (*A–D*) and CD8 (*E–H*) cells for each individual were obtained by gating on CD4 and CD8 positive cells, respectively. The geometric means are similarly plotted (– – –).

trol is preincubated with an excess of unlabelled anti-cytokine antibody. The specifically stained samples are similarly treated with an identical amount of irrelevant isotype matched antibody. After 1 h, identical amounts of directly conjugated anti-cytokine antibody are added to both tubes. As can be seen in Fig. 3, this approach, which we now routinely use, provides a negative control in which specific binding is blocked by an excess of unlabelled anti-cytokine antibody, but in which nonspecific staining remains unaffected relative to the isotype matched control blocked sample.

Further, blocking with unlabelled anti-IL-2 specifically affects IL-2 immunofluorescence but not that of IFN-γ (Fig. 3B), as well as the converse (Fig. 3C). This blocking technique thus consistently reproduces the mean fluorescence intensity of the negative peak of the specifically stained sample (Fig. 3). Such negative controls typically demonstrate 0.01–0.1% false positives, resulting in an order of magnitude greater sensitivity than that of previous reports (Jung et al., 1993; Assenmacher et al., 1994). Thus, we have operationally used the staining of the blocked sample to represent the nonspecific staining inherent in the system; any staining above this level is by definition specific staining for a given cytokine.

3.3. Demonstration of antibody specificity

The ability to effectively block specific staining using either recombinant cytokine or unlabelled anti-cytokine antibody strongly suggests, but does not prove, that the antigen being recognized intracellularly is the cytokine in question. The antibody might crossreact with an intracellular antigen that shares an epitope with the cytokine of interest. Blocking, as performed above, only verifies that the intracellular antigen being recognized displays this

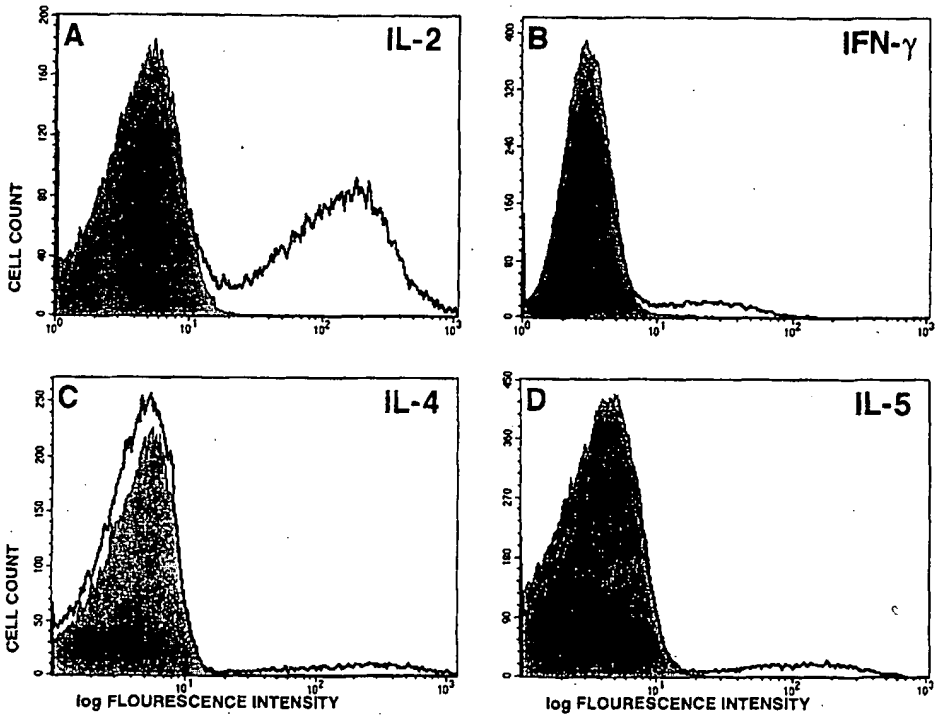

Fig. 2. Recombinant cytokine blocks specific anti-cytokine staining. PBMC from normal (IL-2 and IFN-γ) and eosinophilic donors (IL-4 and IL-5) were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for 6 h and fixed. PE labelled anti-cytokine antibody and anti-CD4 FITC were then incubated with either a molar excess of the corresponding recombinant cytokine (shaded histograms) or an equivalent volume of PBS/BSA (open histograms) prior to staining. Single color histograms were then generated by gating on CD4 positive cells.

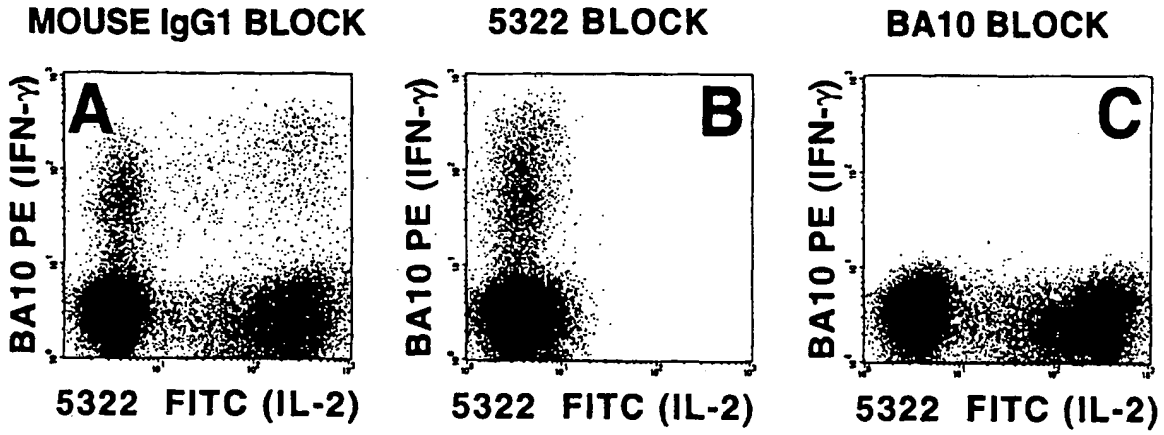

Fig. 3. Unlabelled anti-cytokine mAb blocks specific anti-cytokine staining. PBMC were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for 6 h, fixed, and preincubated with either mouse IgG1 control (*A*); unlabelled 5322 (anti-IL-2) (*B*) or unlabelled BA10 (anti-IFN-γ) (*C*) at 100 μg/ml. Samples were then identically stained with 5322 FITC and BA10 PE.

epitope. To address this question more directly, we used two anti-IL-5 monoclonal antibodies known to recognize different epitopes (clones 5A10 and 39D10) and have demonstrated that both yield the same frequency of positive staining cells (data not shown). To determine if the cells staining with each antibody were the same, three color flow cytometry was next performed using 5A10 FITC, 39D10 PE and anti-CD4 PE/Cy5 (Fig. 4). As shown, there is a population of positive staining cells on the diagonal, demonstrating that cells staining with one anti-IL-5 mAb (5A10) do indeed co-stain with the other mAb (39D10) (Fig. 4A). Blocking performed with unlabelled 5A10 or 39D10 confirmed the specificity of the staining (Fig. 4B and C). That the positive staining cells stain for both IL-5 epitopes in an immunologically specific manner suggests even more strongly that staining is due to specific recognition of IL-5 rather than recognition of a crossreacting antigen.

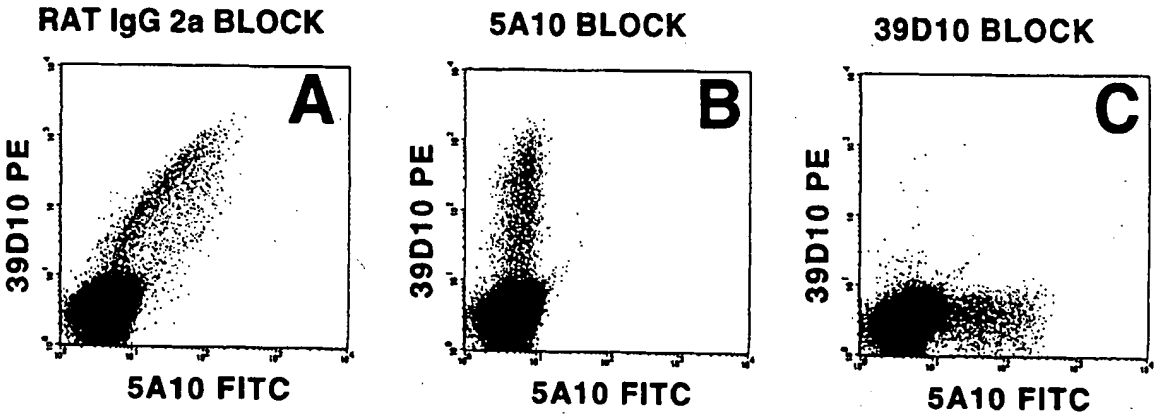

Fig. 4. Demonstration of the specificity of recognition of IL-5. PBMC from an eosinophilic donor were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for 6 h, fixed, and preincubated with either rat IgG2a control (*A*); unlabelled 5A10 (*B*) or unlabelled 39D10 (*C*) at 100 μg/ml. Samples were stained with 5A10 FITC, 39D10 PE and CD4 PE/Cy5; thus differentially staining for two distinct epitopes on the IL-5 molecule. Two color dot plots were generated after gating on CD4 positive cells.

Table 1
Fixed stimulated cells exhibit an extracellular pool of cytokine

Cytokine	Saponin	% positive staining	Mean fluorescence
IL-2	+	41	220
	−	18	59
IFN-γ	+	47	460
	−	7.6	63
IL-4	+	7.9	177
	−	2.3	28
IL-5	+	5.4	348
	−	1.8	42

PBMC from normal (IL-2 and IFN-γ) and eosinophilic donors (IL-4 and IL-5) were stimulated and fixed as per materials and methods and stained with anti-CD3 FITC and the corresponding PE labelled anti-cytokine antibody in the presence (+) or absence (−) of saponin containing buffers. Single color histograms were then generated gating on CD3 positive cells. Statistical gates were set based upon the corresponding unlabelled antibody blocked controls.

3.4. Surface bound cytokine

It has been reported that small but significant numbers of cytokine staining cells are identified when samples are stained without saponin (Jung et al., 1993). We thus examined samples stained with directly labelled anti-cytokine antibodies using identical buffers with or without saponin. We found a considerable variation in the amount of cytokine that was accessible to staining without the use of saponin (Table 1). For IL-2, almost half the number of cells detected using saponin could be detected in its absence. The other cytokines examined exhibited less apparent saponin-independent immunostaining. In all samples examined, the mean fluorescence intensity of the surface stained cells was manyfold less than that of the permeabilized samples.

The saponin independent cytokine staining could result from autocrine or paracrine transfer of cytokine with receptor or membrane association of cytokine, increased accessibility of cytokine to the extracellular environment occurring at the time of exocytosis of a cytokine laden vesicle, or physical trauma to the fixed cells that would leave them permeabilized without the addition of saponin. To address this question, we differentially stained saponin independent and saponin dependent cell associated cytokines. We used FITC and PE labelled anti-cytokine mAbs to differentially stain the two pools of cytokine as per material and methods. When cells were stained in this manner for IL-2, IL-5 and IFN-γ, we found two distinct populations of cytokine staining cells (Fig. 5). A minority of cells found along the diagonal stained for both intracellular (saponin dependent) and extracellular (saponin independent) cytokine. The remaining positive cells stained solely for intracellular cytokine. No cells were detected that stained exclusively for extracellular cytokine.

3.5. IL-5 frequencies in eosinophilic subjects

A high degree of correlation between cytokine flow cytometry and ELISA determinations in T cell

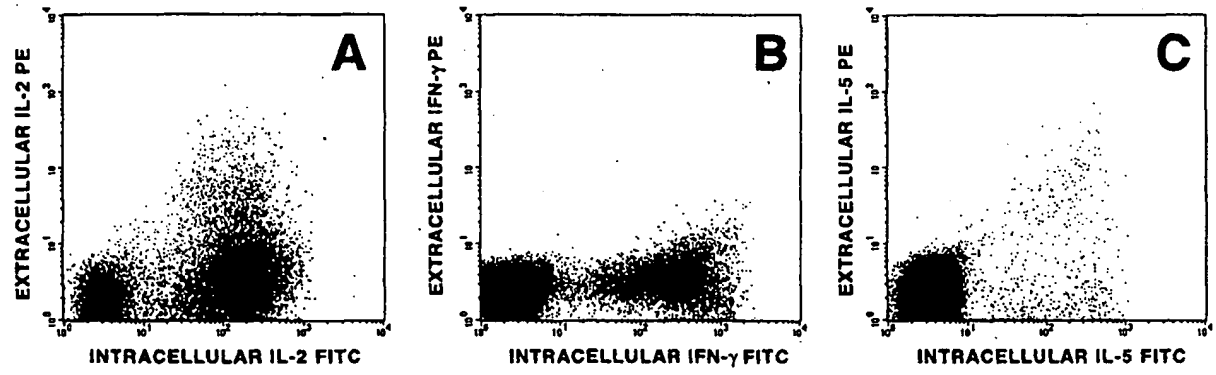

Fig. 5. Differential immunofluorescent staining of extracellular and intracellular pools of cytokine. PBMC were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for 6 h, fixed, and differentially stained for PE labelled extracellular (saponin independent) and FITC labelled intracellular (saponin dependent) pools of IL-2 (*A*), IFN-γ (*B*) and IL-5 (*C*). Two color dot plots were generated after gating on CD4 PE/Cy5 positive cells.

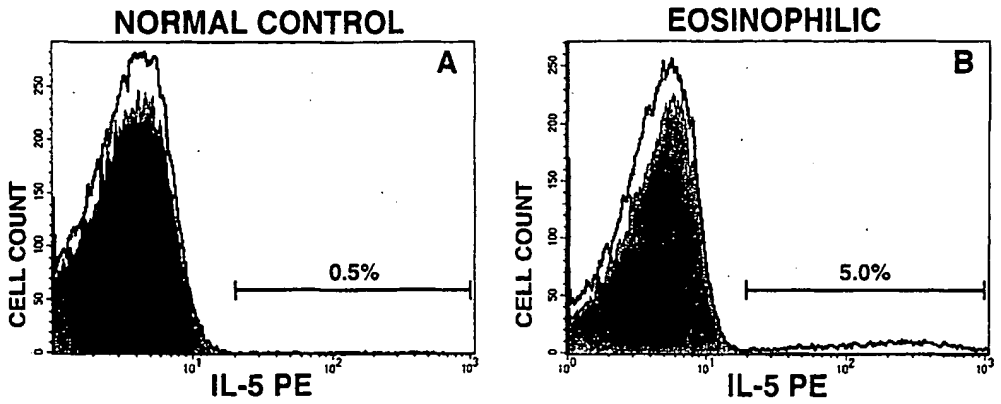

Fig. 6. IL-5 frequencies in a subject with eosinophilia. PBMC from a control subject (*A*) and an eosinophilic subject (*B*) were stimulated with 1 μM ionomycin, 20 ng/ml PMA and 2 μM monensin for 6 h, fixed and preincubated with either rat IgG2a control (open histograms) or unlabelled anti-IL-5 (shaded histograms) at 100 μg/ml. Samples were then stained with anti-CD4 FITC and anti-IL-5 PE. Single color overlay histograms were generated after gating on CD4.

clones and fresh cells has been reported (Jung et al., 1993; Elson et al., 1995). We sought to extend this observation by examining cytokine producing cells from an eosinophilic subject. IL-5 production, as determined by both ELISA and ELISPOT has been shown to be increased in this population (Mahanty et al., 1992). Whereas a normal subject demonstrated 0.5% IL-5 producing CD^+ cells, 5% of $CD4^+$ cells from an eosinophilic subject produced IL-5 (Fig. 6).

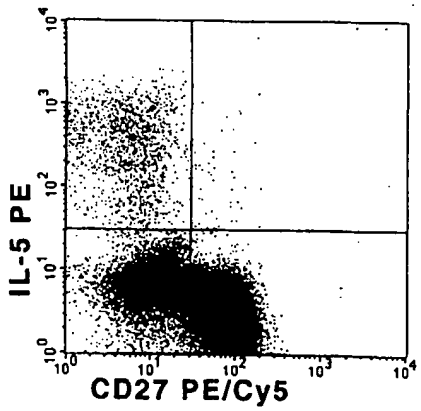

Fig. 7. Subpopulation analysis of cytokine production by flow cytometry. PBMC from an eosinophilic subject were stimulated and fixed as per Materials and methods and stained with anti-CD27. The cells were washed, stained with goat anti-mouse PE/Cy5, washed again, blocked with 2% mouse serum and preincubated with either rat IgG2a control or unlabelled anti-IL-5 at 100 μg/ml. They were then stained with anti-CD4 FITC and anti-IL-5 PE. Two color dot plots were generated after gating on CD4 positive cells. Statistical gates were set based upon the unlabelled antibody blocked control.

3.6. Analysis of rare cell populations

The ability to gate upon cell populations of interest is also a strength of cytokine flow cytometry. Lymphocyte subpopulations, which by themselves are not easily physically purified, may thus be analyzed by gating on specific cell surface markers. CD27, a member of the TNF receptor superfamily, is expressed on approximately 90% of peripheral blood CD4 cells in normal subjects. The fraction of $CD4^+$, $CD27^-$ cells is increased in eosinophilic subjects, and this fraction demonstrates enhanced production of IL-4 and IL-5 (Elson et al., 1994; Elson et al., 1995). To evaluate the utility of gating for a small subpopulation, we simultaneously stained stimulated, fixed PBMC from an eosinophilic donor for CD4, CD27 and IL-5. CD4 positive cells were gated upon, and a 2 color dot plot was created by plotting CD27 vs. IL-5 fluorescence. Greater than 95% of IL-5 staining cells were found within the $CD27^-$ subset, despite the fact that it accounted for only 25% of the total number of CD4 cells (Fig. 7).

4. Discussion

In this report, we describe the modification of an established fixation and permeabilization technique

for the detection of intracellular cytokines (Sander et al., 1991). With these modifications and using directly conjugated anti-cytokine monoclonal antibodies, we have been able to decrease nonspecific staining in the negative controls to below 0.1%, allowing the detection of IL-5 producing cells (Figs. 5–7), which generally comprise less than 1% of CD4 cells and are thus below the threshold of detection of previous reports. Fixation, which is necessary to immobilize intracellular cytokine, greatly increases nonspecific staining. This is especially evident when staining with secondary or biotinylated antibodies. The use of non-fat dried milk in *both* blocking and staining buffers as well as the use of directly conjugated mAbs decreased nonspecific binding to an acceptable level. As such, the level of fluorescence due to nonspecific binding is only slightly greater than that of cellular autofluorescence. As shown in Figs. 3, 4 and 6, the addition of excess unlabelled anti-cytokine mAb causes no shift in the negative peak relative to the addition of isotype matched control antibody. We also noted that PE conjugates generally provided superior signal to noise ratios compared to FITC labelled mAbs.

Another major advantage of directly labelled mAbs is in the generation of unbiased statistical markers. Previous studies have used either isotype matched, secondary antibody only or recombinant cytokine blocked controls (Jung et al., 1993; Assenmacher et al., 1994). We have routinely found that the former two controls are inconsistent estimates of nonspecific binding. This is especially apparent in the detection of IL-4 and IL-5, where a small movement of statistical gates can greatly affect the frequency of positive cells. Blocking with recombinant cytokine provides an excellent negative control (Fig. 2), but requires large amounts of expensive cytokine. By using directly labelled mAbs, we are able to block specific binding by preincubating the cells in an excess of unlabelled anti-cytokine antibody (Figs. 3 and 6). We now use these unlabelled antibody blocked negative controls as our routine method to place statistical markers differentiating positive from negative staining. This yields a negative control that is specific, inexpensive and has low noise.

We have used this blocking technique to demonstrate the specificity of staining. Blocking with either unlabelled mAb or recombinant cytokine provides a degree of reassurance that the fluorescence observed is due to that cytokine. However, it does not address the possibility of antibody crossreacting with another intracellular antigen or cytokine. We thus performed differential 2 color staining with FITC and PE labelled mAbs recognizing two different epitopes on the IL-5 molecule. We found the same cells stained specifically for both mAbs, making it extremely unlikely that there is immunological crossreactivity with both epitopes on the IL-5 molecule (Fig. 4).

We have chosen to study cells after 6 h of stimulation, as this time point was optimal for frequencies of IL-4 and IL-5 producing cells; for IL-2 and IFN-γ, frequencies greater than 80% of maximal were found at this time point (Fig. 1). Examination of other cytokines and stimuli may require different time points. IL-2 was the dominant cytokine produced by CD4 cells, whereas IFN-γ predominated in the CD8 response (Fig. 1A, E, B, F). Although IL-4 and IL-5 frequencies were generally higher in the CD4 subset, substantial numbers of IL-4 and IL-5 producing CD8 T cells were noted, supporting recent work describing 'Th2' CD8 cells (Seder and LeGros, 1995).

Minimal staining has been noted when saponin is left out of buffers (Jung et al., 1993). Using direct conjugates, we found a greater amount of such surface staining, albeit with low fluorescence intensity (Table 1). This discrepancy appears to be due to the greater signal to noise ratio of the direct conjugates we used. Much of the low intensity fluorescence staining we observed may have been lost in the negative peak in the previous study. Using FITC and PE labelled anti-cytokine mAbs we differentially stained the extracellular (saponin independent) and intracellular (saponin dependent) cytokine pools (Fig. 5). All extracellular cytokine was associated with cells that also stained for intracellular cytokine. We found no change in this saponin independent staining when the anti-IL-2 receptor mAb anti-Tac was added to the cell culture (data not shown), demonstrating that this staining is not mediated by uptake via the IL-2 receptor. This further confirms that under these conditions there is no detectable passive transfer of cytokine.

This lack of passive transfer from producer cells to target cells is not a surprising finding, given the addition of monensin from the start of culture. Mon-

ensin inhibits cytokine secretion, increasing the quantity of intracellular cytokine and thus enhancing fluorescence intensity. The reciprocally decreased cytokine secretion may act to decrease passive transfer caused by secreted cytokines. Use of monensin has to be tempered with its' toxicity in cultures of greater than 16 h duration (Jung et al., 1993). Although monensin has been reported to increase monocyte IL-1β production (Orlinska and Newton, 1992) there are no similar reports of its induction of T cell cytokines.

We have demonstrated the correlation of cytokine flow cytometry to supernatant ELISA (Elson et al., 1995). We have extended that observation by examining the frequencies of IL-5 producing CD4 cells in a normal and in an eosinophilic subject. The eosinophilic subject showed a ten-fold greater frequency of IL-5 producing CD4 T cells (Fig. 6). Thus, cytokine flow cytometry is able to correlate an enhanced frequency of cytokine producing cells to a specific pathologic state.

As the questions asked by immunologists have become more sophisticated, the methods employed to address these questions have also become more involved. Because of the heterogeneity of the T cell population, inquiry into the role of specific subpopulations has required ever more elaborate schemes to purify these subpopulations for study. The ability of the flow cytometer to electronically gate on specific subpopulations of interest has provided us with a powerful tool to investigate such subpopulations without labor intensive cell purifications. To this end, we have established, using flow cytometry, that one can isolate cytokine production within the minority $CD4^+CD27^-$ subpopulation (Fig. 7); clearly demonstrating that virtually all IL-5 is produced by the $CD27^-$ subset.

In summary, we have developed a cytokine flow method that is easy to perform and is more sensitive than previously reported methods. Blocking with unlabelled antibody controls allows the specificity of staining to be demonstrated on a routine basis. We have proven that the antigen being recognized intracellularly is indeed the cytokine of interest. Furthermore, we have demonstrated the utility of this technique to examine the cytokine production of a small T cell subpopulation without prior cell purification. We believe with these improvements, cytokine flow cytometry becomes a practical tool for immunologists.

Acknowledgements

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Direct Demonstration of Cytokine Synthesis Heterogeneity Among Human Memory/Effector T Cells by Flow Cytometry

By Louis J. Picker, Manoj K. Singh, Zoran Zdraveski, John R. Treer, Sharr L. Waldrop, Paul R. Bergstresser, and Vernon C. Maino

The array of cytokines produced by T cells in effector sites is a primary means by which these cells mediate host defense. It is well recognized that cloned T cells are heterogeneous with regard to cytokine synthesis and, thus, in their ability to mediate specific immune responses, but the extent to which the patterns of cytokine secretion observed in cloned cells reflect actual populations of memory/effector T cells existing in vivo is largely unknown. Here, we report our findings using a multiparameter flow cytometric assay that allows simultaneous determination of an individual T-cell's ability to produce multiple cytokines and its phenotype after only short (4 to 8 hours) in vitro incubation with an activating stimulus and the secretion inhibitor Brefeldin A. This assay shows a rapid accumulation of interleukin-2 (IL-2), IL-4, and γ-interferon (γ-IFN) in the cytoplasm of CD4+ cells after stimulation with either accessory cell-independent (phorbol 12-myristate 13-acetate [PMA] + ionomycin [I]) or accessory cell-dependent (staphylococcal enterotoxins [SE] A and B) T-cell–activating stimuli. Further analysis showed that production of γ-IFN and IL-4 is predominantly, if not exclusively, restricted to the CD45RO^{high} memory/effector T-cell subset, whereas IL-2 may be produced by both the CD45RO^{high} and CD45RO^{low} subsets. Simultaneous determination of IL-2 and γ-IFN production among CD45RO^{high}/CD4+ T cells showed distinct subsets that produce each of these cytokines alone (an average of 30% for IL-2 alone, 8% for γ-IFN alone), both (16%), or neither (46%). Similar analyses with the small IL-4–producing memory/effector T-cell subset (only 4.3% of total CD4+/CD45RO^{high} T cells) showed that an average of 51% of these IL-4–producing cells also synthesize IL-2, 23% synthesize only IL-4, 16% synthesize all three cytokines, and 9.6% synthesize IL-4 and γ-IFN. These patterns of cytokine synthesis were found to be similar with both PMA + I and SEA/SEB stimulation and were observed in both peripheral blood memory/effector CD4+ T cells and in T cells of similar phenotype obtained from cutaneous delayed-type hypersensitivity sites. Taken together, these data strongly support the in vivo existence of human memory/effector T-cell subsets with "preprogrammed" cytokine synthesis potential, although they suggest that these subsets may be more complex than originally proposed in the TH1/TH2 hypothesis.

THE MANIFESTATION OF immunity, either protective or pathologic, critically depends on the functional activity of the so-called memory/effector T-cell subset, characterized in the human by its CD45RA^{low}/RO^{high} phenotype. Indeed, only these previously activated T cells appear to have the capability of efficiently homing to and localizing within sites of T-cell effector function, including all extralymphoid sites of inflammation (eg, lung, skin, synovium, and so on) and the unique lymphoid microenvironment of the germinal center.[1,2] However, memory/effector T cells are not uniform in their functional potential. Similar to the immune reactions they initiate, these cells manifest remarkable heterogeneity, both in terms of their ability to extravasate in a particular site and to manifest effector function therein.[1-9] For example, we have described and characterized a subset of CD45RA^{low}/RO^{high} T cells that are selectively targeted to skin,[3,4] and subsets thought to be targeted to other tissues have been described as well.[1-4] However, perhaps the most investigated functional heterogeneity shown by memory/effector T cells is their cytokine synthesis potential.[1,5-10] Detailed studies of cloned CD4+ T cells in both mice and humans have suggested that the ability to synthesize and secrete many key effector cytokines, including interleukin-2 (IL-2), IL-4, IL-5, and γ-interferon (γ-IFN), is restricted to a fraction of the overall memory/effector population. Indeed, the finding of predominant patterns of cytokine secretion among T-cell clones has led to the proposal that CD4+ effector T cells may be generally separated into TH1 (IL-2– and γ-IFN–producing) and TH2 (IL-4– and IL-5–producing) subsets.[5-10]

Even though the cytokine synthesis potential of individual, genuine (ie, noncloned) effector T cells is largely unknown, the TH1/TH2 terminology has become so well accepted that it is routinely extended to complex in vivo immune responses (so that responses with a predominance of γ-IFN– or IL-4–producing T cells are termed "TH1" or "TH2" responses, respectively). Because recent evidence has strongly suggested that the ability of memory/effector T cells to produce a given cytokine is heavily influenced by the microenvironmental conditions present at the time of T-cell activation,[8-12] it is plausible that the long-term in vitro culture required for cloning T cells may shape the cytokine synthesis phenotype of the resulting clones. Thus, the patterns of cytokine secretion observed in such clones may not be representative of all physiologically relevant memory/effector T-cell subsets. Given the clear importance of understanding the entire physiologic range of T-cell functional heterogeneity, we sought to assess the cytokine (γ-IFN, IL-2, and IL-

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4) synthesis capabilites of normal, freshly isolated, human memory/effector T cells on a single-cell basis. These studies were based on the pioneering methodological report of Jung et al,[13] who first showed the feasibility of using intracytoplasmic staining and flow cytometry to assess T-cell cytokine production. Here, we improve on these methods and combine them with sophisticated multiparameter analysis techniques to simultaneously determine the cytokine production capabilities of precisely defined subsets of human peripheral blood (PB) and inflammatory site T cells after only short periods (4 to 8 hours) of in vitro activation with mitogen or superantigen. Our results confirm an extensive heterogeneity in the ability of memory/effector T cells to produce these three cytokines in response to both accessory cell-dependent and accessory cell-independent stimuli and show reproducible patterns of cytokine secretion by these cells. The direct visualization of T-cell effector responses afforded by this flow cytometric approach allows an unparalleled ability to determine the functional potential of phenotypically distinct T-cell subsets and, thus, the opportunity to evaluate the participation of these subsets in human immune responses.

MATERIALS AND METHODS

Monoclonal antibodies (MoAbs). MoAbs Leu4 (CD3; fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll protein [PerCP]), Leu3a (CD4; FITC, PE, PerCP), Leu2a (CD8; FITC, PE, PerCP), Leu45RO (CD45RO; unconjugated, PE), Leu18 (CD45RA; unconjugated), Leu23 (CD69; FITC, PE), G1CL (mouse IgG1 control; FITC, PE, PerCP), and G2GL (mouse IgG2 control; FITC, PE, PerCP) were obtained from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA). Purified MoAbs B-G5 (antihuman IL-2; mouse IgG1) and B-B1 (antihuman γ-IFN; IgG1) were purchased from Biosource International (Camarillo, CA) and were conjugated to PE or FITC by the reagent laboratories at BDIS. The antihuman IL-4 MoAb IL-4I (mouse IgG1) and a mouse IgG1 control reagent were purchased from Pharmingen (San Diego, CA). A series of mouse antihuman cytokine MoAbs (all IgG1) from the research labs of R & D Systems (Minneapolis, MN) were also used in this study (largely to confirm the staining specificity of the above-listed reagents), including clones no. 3010.2-4 and 3011.1 (both anti-IL-4), no. 25723.11 (anti-γ-IFN), and no. 5302.111, 5338.111, and 5344.111 (all anti-IL-2). The unconjugated anticytokine MoAbs were visualized with a Tricolor-conjugated goat antimouse IgG1 second stage obtained from Caltag (South San Francisco, CA).

Cell preparation. PB mononuclear cells (PBMCs) obtained from normal donors were isolated from heparinized venous blood by density-gradient sedimentation over Ficoll-Hypaque (Histopaque; Sigma Chemical Co, St Louis, MO). Cells were then washed 3 times in Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY) and resuspended in medium appropriately for cell culture, immunofluorescence staining of freshly isolated cells, or futher purification. Purified T cells (>95% CD3+) were isolated from the PBMC preparations by negative selection using R & D Systems T-cell purification columns, as described by the manufacturer. Purified whole virgin (≥95% $CD45RO^-$), whole memory/effector (≥95% $CD45RO^+$), or $CD4^+$ memory/effector (≥95% $CD4^+$ and ≥95% $CD45RO^+$) T cells were isolated from T-cell preparations via 2 to 3 rounds of negative panning using the Leu45RO, Leu 18, and Leu2a + Leu18 MoAbs, respectively, as previously described.[14] In some experiments, accessory cell (monocyte) reconstitution was required to allow purified T-cell preparations to respond to superantigens (see below). For these experiments, the culture wells to be used with the purified populations were preseeded with PBMCs at 0.5×10^6 cells/mL in complete media and allowed to incubate for 1 hour at 37°C, after which the culture wells were extensively washed to remove nonadherent cells (leaving >95% monocytes) and then were seeded with purified T-cell populations (see below).

Leukocyte preparations from delayed-type hypersensitivity (DTH) sites in skin were obtained as previously described.[15] Briefly, common skin-test antigens (Ags; eg, mumps Ag) were injected intradermally into the normal forearm skin of sensitized volunteers before the placement of suction blisters 18 to 24 hours later. (Clinical evidence of a delayed hypersensitivity reaction usually appeared at about 24 hours, peaking at 40 to 50 hours.) Leukocyte-containing blister fluid was withdrawn at successive 24-hour intervals after blister placement for up to 92 hours. (Fluids were completely evacuated at each time point, with natural refilling occurring between samplings.) Collected leukocytes were washed by centrifugation and processed immediately for the immunofluorescent cytokine production assays. The results presented in this study represent the characterization of T cells taken from the blister fluid sample correlating with the peak of the observed DTH response.

Flow cytometric cytokine production assays. Flow cytometric assessment of T-cell cytokine production is based on the stimulation of T cells in the presence of a pharmacologic inhibitor of secretion, followed by cell fixation and permeabilization, and then intracytoplasmic staining of acccumulated cytokines. This technique was originally described by Jung et al[13] but has been significantly modified in these studies. These changes have both simplified the staining procedure and considerably enhanced the sensitivity and reproducibility of the cytokine detection (data not shown). In our protocol, Brefeldin A (BFA), a relatively nontoxic but potent inhibitor of intracellular transport,[16] was used to block the cytokine secretion stimulated by both accessory cell-independent (phorbol 12-myristate 13-acetate [PMA] + ionomycin [I]) and accessory cell-dependent (the bacterial superantigens, staphylococcal enterotoxin [SE] A and SEB)[17,18] T-cell agonists. In preliminary experiments, BFA (10 μg/mL final concentration) completely blocked the surface appearance of the activation Ag CD69[19] on 4-hour PMA + I-stimulated T cells without any loss of viability (viability maintained for for up to 18 hours). Importantly, BFA did not interfere with early activation events, because the level of intracytoplasmic CD69 staining (see below) shown by PMA + I-activated, BFA-treated T cells was almost identical to the level of surface CD69 staining on non-BFA-treated, similarly activated T cells (data not shown). Preliminary experiments also indicated that maximal accumulation of intracellular cytokine occurred after 4 hours of BFA-inhibited PMA + I stimulation and after 6 to 8 hours of BFA-inhibited superantigen stimulation. (Cytokine accumulation plateaued and then decreased with longer periods of BFA exposure.) Thus, T cells (without accessory cells for PMA + I stimulation; with accessory cells for superantigen stimulation) were cultured in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Sterile Systems, Inc, Logan UT), 20 mmol/L HEPES buffer (GIBCO), 100 U/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), and 2 mmol/L L-glutamine (GIBCO) with or without the T-cell stimulants PMA + I (5 ng/mL and 1 μmol/L, respectively; Sigma), or the superantigens SEA and SEB (100 ng/mL of each; Toxin Technology, Sarasota, FL). BFA was added 4 hours (PMA + I) or 6 to 8 hours (SEA + SEB) before harvest so that, in kinetic experiments, the period of secretion inhibition and, thus, cytokine accumulation was for 4- or 6-hour windows of time (eg, 0 to 4 hours, 14 to 18 hours, 40 to 44 hours, and so on).

After harvesting, cells (0.1 to 0.5×10^6/test) were washed with cold HBSS, stained (if appropriate) on the cell surface with an MoAb-fluorochrome conjugate (IgG2 MoAbs only, in this case Leu45RO), and then were simultaneously fixed and permeabilized

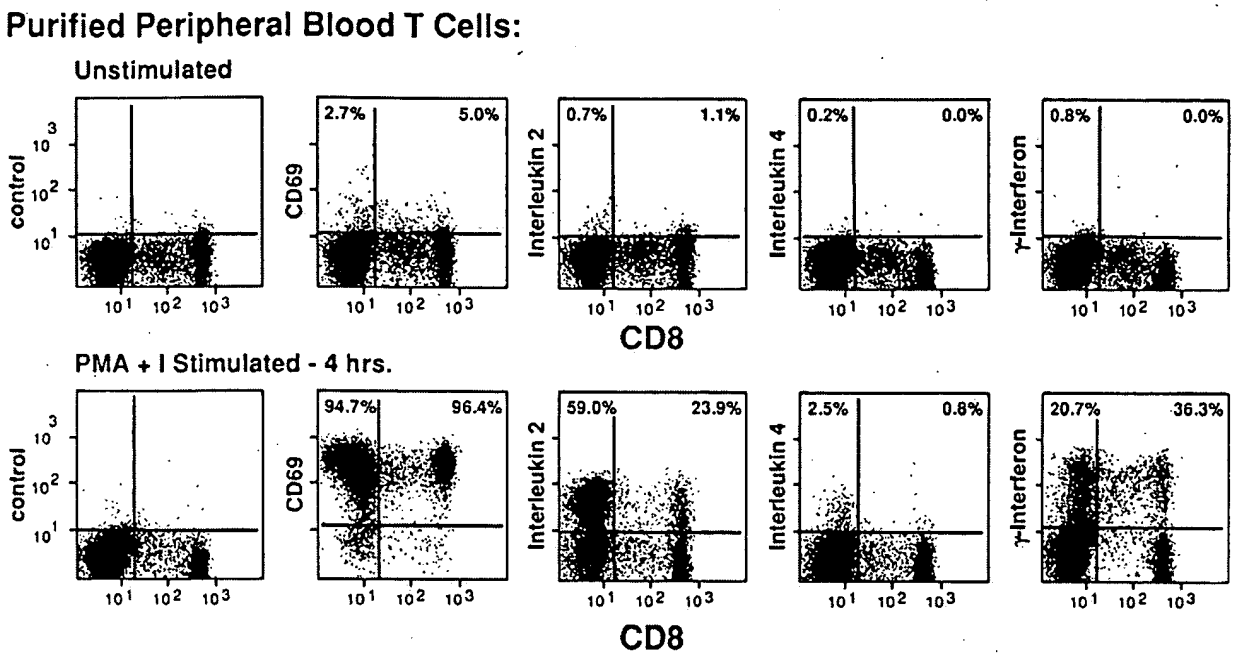

Fig 1. Flow cytometric detection of cytokine synthesis among purified PB T cells stimulated with PMA + I. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of the secretion inhibitor BFA and then compared with unstimulated T cells for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN) or the activation Ag CD69 versus CD8. A total of 10,000 events, gated on viable cells using light scatter criteria, are shown in each plot. The percentages in the upper left and right corners of each plot represent the net percentage (%) of positive (+) cells (ie, after subtracting background) among the $CD8^-$ and $CD8^+$ subsets, respectively. As discussed in the Materials and Methods section, the $CD8^-$ population is essentially equivalent to the $CD4^+$ subset in these experiments. Note that the vast majority of the PMA + I-treated T cells are highly positive for the activation Ag CD69, indicating that the lack of production of a particular cytokine by a given cell was not likely due to incomplete activation.

in HBSS with 10 mmol/L HEPES buffer, 4% paraformaldehyde, and 0.1% saponin (all from Sigma) for 10 to 15 minutes at 4°C. After washing 2 times with cold Dulbecco's phosphate-buffered saline (dPBS) containing 0.1% bovine serum albumin, cells were stained as previously described[15,20] for surface Ag immunofluorescence. Continued or additional permeabilization was not necessary for optimal staining. Briefly, cells were incubated successively with the following reagents (all at 0 to 4°C) with washing in dPBS/bovine serum albumin in between: (1) an appropriately titred unconjugated anticytokine MoAb (usually 0.1 to 0.4 μg/test for 30 minutes); (2) the Tricolor-conjugated goat antimouse IgG1 second stage in dPBS with 5% normal human serum (30 minutes); (3) 0.4 mL/test of 10% normal mouse serum (10 minutes; for blocking); and (4) 1 to 2 conjugated MoAbs against additional cytokines or other Ags (CD4, CD8, and/or CD69).

The CD4 and CD8 MoAbs delineated identical populations whether they were used before or after permeabilization. However, because of the previously documented rapid and complete downmodulation of CD4 in response to phorbol esters,[21] CD4 MoAbs could not be used to delineate the $CD4^+$ T-cell subset after more than 4 hours of PMA + I stimuluation. Because CD8 expression diminishes only slightly after this treatment, and because multiparameter staining of the unstimulated purified T-cell preparations used in our study showed that the vast majority of $CD8^-$ cells were indeed $CD4^+$ (not shown), CD8 MoAbs alone could effectively differentiate the $CD4^+$ and $CD8^+$ subsets. Therefore, in the experiments examining PMA + I-stimulated purified T cells, the $CD8^-$ subset was considered equivalent to the $CD4^+$ subset. CD4 downmodulation does not occur with superantigen stimulation; therefore, the CD4 subset could be directly identified in these experiments. CD69 expression was used in these studies as an indicator of activated cells.[19] In BFA-treated cells, CD69 upregulation is confined to the cytoplasm, requiring that CD69 staining be performed after permeabilization (see above). In contrast, BFA protected the cell surface from activation-induced changes in CD45RO expression, allowing delineation of the original virgin and memory/effector subsets only if staining was performed before permabilization. Because continuous BFA was required for this effect and because continuous BFA for longer than 8 hours results in diminshing cytokine accumulation, this technique could only be used for the 0- to 4-hour or 0- to 6-hour time points.

Five-parameter analysis was performed on a FACSort flow cytometer (BDIS) using FITC, PE, and PerCP or Tricolor (PE/Cy5 tandem) as the three fluorescent parameters. Methods of cytometer setup and data acquisition have been described previously.[20] List-mode multiparameter data files (each file with forward scatter, side scatter, and three fluorescent parameters) were analyzed using the PAINT-A-GATE^{Plus} program (BDIS). Negative control reagents were used to verify the staining specificity of experimental antibodies and as a guide for setting markers to delineate positive and negative populations. All percentages listed in the figures and text represent net percent positive, after subtraction of background. All analyses were performed using a light scatter gate designed to include only viable lymphocytes. In some analyses, additional live gating based on CD4 or CD8 reactivity or CD69 reactivity was performed to enhance the sampling of small populations. Preliminary experiments using this flow cytometric cytokine assay on two well-characterized human TH1 clones and on IL-4 (versus control) transfectants showed an excellent qualitative correlation between the flow cytometric results and the level of overall cytokines measured by conventional techniques (immunoassay, analysis of mRNA; data not shown), similar

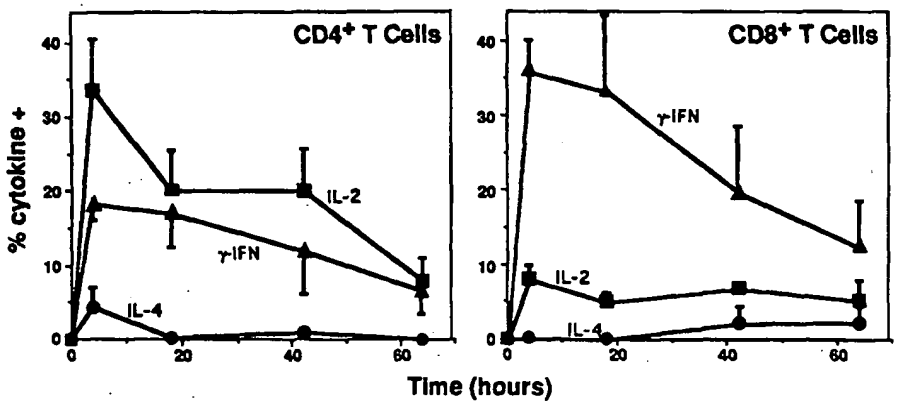

Fig 2. Flow cytometric characterization of the kinetics of cytokine synthesis among purified PB T cells stimulated with PMA + I. Purified PB T cells were stimulated with PMA + I for 4, 18, 42, and 66 hours (with BFA added during the final 4 hours of culture to inhibit cytokine secretion) and then analyzed for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN) versus CD8. The results presented are the mean percentage (%) of cytokine positives (± SEM) for the CD4+ (CD8−) and CD8+ T-cell subsets in 3 independent experiments. In these experiments, essentially 100% of T cells manifested CD69 expression by the 18-hour time point, indicating complete activation.

to previously reported results.[13] Moreover, identical patterns of reactivity were observed with at least two independent MoAb clones for each cytokine. The data presented in the figures are representative of 3 to greater than 10 separate experiments.

RESULTS

Initial studies focused on the flow cytometric characterization of the cytokine production capabilities of purified PB human T cells activated by the accessory cell-independent stimulus of PMA + I in the presence of BFA. Significant cytokine reactivity was not detected within fresh, unstimulated T cells but was dramatically upregulated (along with the activation Ag CD69) after as little as 4 hours of incubation with this potent activating stimulus (Fig 1). This response was not apparent or was markedly diminished with (1) in vitro incubation in the absence of an activating stimulus (with or without BFA), (2) appropriate activation in the absence of secretion inhibition (ie, the inclusion of BFA with the PMA + I), or (3) the lack of cell permeabilization before staining (data not shown). The absolute requirement for permeabilization (ie, the lack of specific signal after cell surface staining), along with the inability of exogenously added cytokine to produce a signal using our staining protocols (data not shown), indicates that T-cell binding to soluble cytokine in the culture medium did not contribute to the observed responses. Cytokine synthesis clearly preceded blastogenesis, because, at the 4-hour time point, the vast majority of cytokine-producing cells still manifested the light scatter properties of small lymphocytes (not shown). Significantly, despite the fact that essentially all PMA + I-treated T cells showed marked upregulation of CD69 (see Fig 1), which is consistent with universal activation, only a subset of T cells produced detectable cytokine at any given time point (Figs 1 and 2). Both the CD4+ (CD8−) and CD8+ subsets participated in this cytokine response, with more CD4+ T cells producing IL-2 than CD8+ T cells, and vice versa for γ-IFN. Among CD4+ cells, IL-2 production usually peaked in the 0- to 4-hour time period, with a sharp partial decrease at the 14- to 18-hour time points and a slow decrease thereafter. Peak levels of γ-IFN by CD4+ T cells and of both γ-IFN and IL-2 by CD8+ T cells were achieved in the first 4 hours of stimulation, with a very slow decrease in the numbers of producing cells over the next 3 days. Among CD4+ T cells, IL-4 production was transient (usually identified in 0- to 4-hour time period only) and was only observed in a small subset of these cells (overall, mean ± SEM = 2.7% ± 0.6% for 18 different experiments). IL-4 was usually detected in even fewer (usually <1%) CD8+ T cells at the 4-hour time point but, in some experiments, reappeared in a slightly larger subset of CD8+ cells after 40 hours of stimulation (Fig 2).

We next assessed the ability of the accessory cell-dependent bacterial superantigens SEA and SEB to stimulate detectable T-cell cytokine responses. Because these superantigens are known to stimulate T cells in a Vβ-specific manner (eg, Vβ1.1, Vβ5, Vβ6, Vβ7.3, and Vβ9.1 for SEA; Vβ3, Vβ12, and Vβ17 for SEB)[22] and, thus, only activate a subset of the overall T-cell population, we simultaneously evaluated CD69 expression along with cytokine synthesis and CD4/8 phenotype so as to determine the percentage of cytokine-producing cells within the activated CD4+ or CD8+ T-cell populations. As shown in Fig 3, only the CD69+ T cells synthesized and accumulated significant intracellular cytokine, allowing us to gate on the CD69+ cells to determine the extent to which SEA-/SEB-responsive cells produce these cytokines. A representative experiment comparing the participation of (CD69-gated) CD4+ versus CD8+ T cells in an SEA-/SEB-induced cytokine response is shown in Fig 4. Similar to what was observed with PMA + I, more CD4+ T cells produce IL-2 than CD8+ T cells, whereas the reverse was true for γ-IFN. However, the overall number of cells manifesting an IL-2 or γ-IFN response to SEA/SEB was less than that observed for PMA + I (even with analysis restricted to the activated population). This was not apparent for IL-2–producing CD4+ cells, which were twofold to threefold fewer in number (see below). In contrast, the percentage

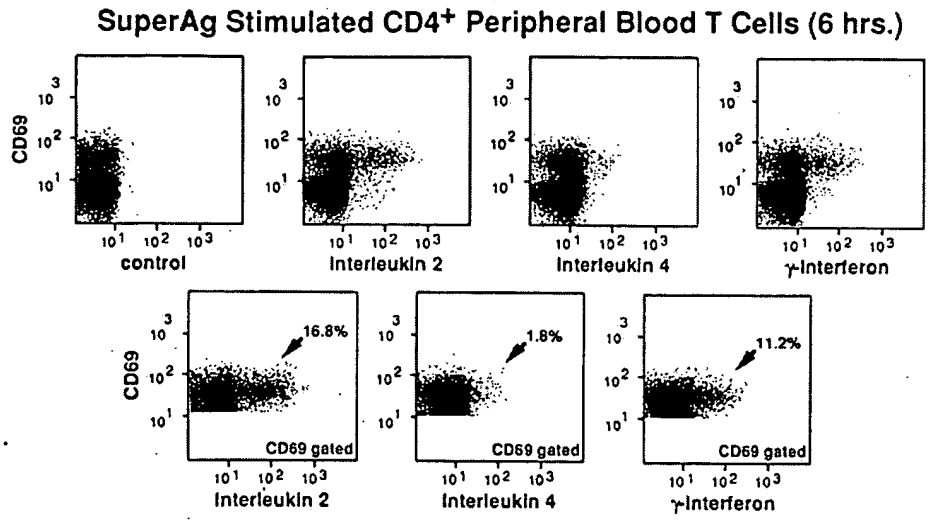

Fig 3. Flow cytometric characterization of cytokine synthesis by superantigen-stimulated PB T cells; evaluation per total activated T cells. PBMCs were stimulated with the bacterial superantigens SEA + SEB for 6 hours in the presence of BFA and then stained intracellularly for (1) IL-2, IL-4, γ-IFN, or an isotype-matched control; (2) CD4; and (3) the rapid activation Ag CD69. A total of 15,000 events gated on CD4 expression (top panel) or 7,500 to 8,000 events gated on CD4 and CD69 expression (bottom panel) are shown. Note that cytokine synthesis is restricted to the activated CD69$^+$ subset (which in this experiment, comprise 20.9% of CD4$^+$ cells at 6 hours). Thus, gating on CD69 allows determination of the cytokine-producing cells as a fraction of the activated population only (16.8%, 1.8%, and 11.2% for IL-2, IL-4, and γ-IFN, respectively, in this experiment). Such gating greatly increases the accuracy and reproducibility of cytokine-producing cell quantitation after stimulation with subset-restricted activating agents (such as superantigen), because it (1) corrects for differences in the number of T cells responding to these agents and (2) allows collection of more positive events (ie, cytokine-producing cells).

of IL-4–producing T cells induced by SEA/SEB (after CD69 gating) was as large or larger than that induced by PMA + I (note particularly the relatively increased IL-4 response in the CD8$^+$ subset). SEA/SEB-stimulated responses for all three cytokines were transient (peak responses always in the 0- to 6-hour time period), despite the fact that the mean percentage (± SEM; n = 4) of CD69$^+$ cells increased from 17.6% ± 1.7% after 6 hours of SEA/SEB stimulation to 51.1% ± 5.5% after 18 hours of stimulation.

Previous studies have established that the so-called virgin and memory/effector subsets of CD4$^+$ T cells, delineated by differential expression of the CD45RA and RO isoforms,

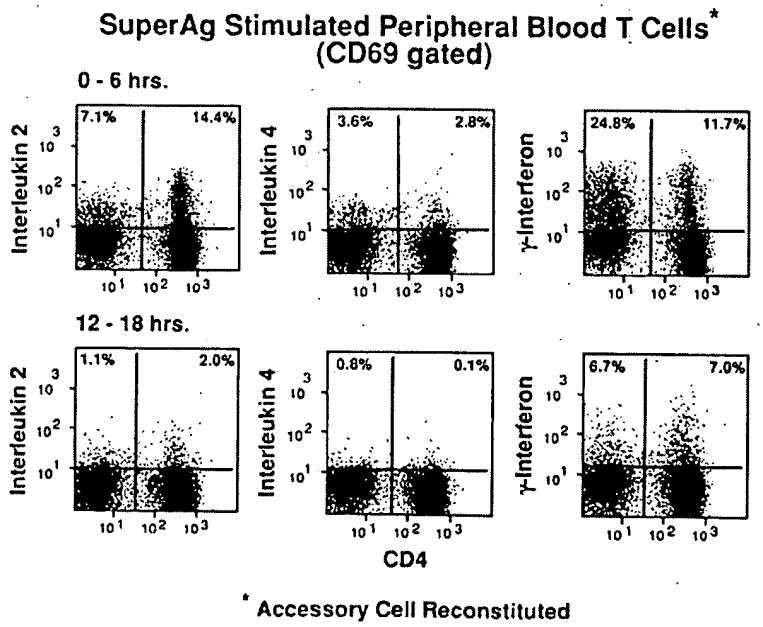

Fig 4. Flow cytometric characterization of cytokine synthesis patterns among superantigen-stimulated PB T cells. Purified PB T cells were reconstituted with monocytes and stimulated with the bacterial superantigens SEA + SEB for 6 and 18 hours (with BFA added during the final 6 hours of culture). These cells were then assessed for their correlated expression of intracellular cytokine (IL-2, IL-4, γ-IFN), CD4, and CD69 and analyzed after gating on the CD69$^+$ T-cell subset, as shown in Fig 3 (10,000 events in each plot). The percentages in the upper left and right corners of each plot represent the net percentage (%) of positive (+) cells among the CD4$^-$ and CD4$^+$ subsets, respectively. Because the original T cells are highly purified, the CD4$^-$ population is essentially equivalent to the CD8$^+$ subset in these experiments (the few nonadherent monocytes present at the time of harvest are eliminated by appropriate gating on light scatter parameters).

differ markedly in cytokine gene expression and secretion. In general, the putative virgin T-cell subset expresses IL-2 only after appropriate stimulation, whereas the putative memory/effector subset expresses all three cytokines.[23-26] Our multiparameter flow cytometric analysis confirms these data on a single-cell basis. Figure 5 shows a representative experiment for PB $CD4^+$ T cells after 4 hours of stimulation with PMA + I. Note that $CD4^+$ T cells with a surface $CD45RO^{high}$ phenotype include the vast majority of T cells producing detectable levels of γ-IFN and IL-4. About half of this subset produces IL-2 as well, but IL-2 is also produced by about 25% of $CD45RO^{low}$ putative virgin T cells. Significantly, among SEA/SEB-stimulated $CD4^+$ T cells, production of all three of these cytokines was restricted to the $CD45RO^{high}$ subset (data not shown), likely accounting for the above noted pronounced decrease in IL-2-producing $CD4^+$ cells after this stimulus, as compared with PMA + I stimulation.

It is possible that the differences observed in PMA + I-induced γ-IFN production by the CD45RO-defined, $CD4^+$ T-cell subsets in Fig 5 are a function of delayed kinetics of γ-IFN production by the $CD45RO^{low}$ subset. (This possibility cannot explain the observed differential IL-4 production, because significant IL-4 production is only present in the 0- to 4-hour time period.) To address this issue, we determined γ-IFN and IL-2 production by CD45RA/RO-defined $CD4^+$ T-cells subsets during the 14- to 18-hour and 36- to 40-hour time periods. Because continuous BFA cannot be used for these longer incubation periods (see Materials and Methods section), we purified $CD45RA^+(RO^-)$ and $CD45(RA^-)RO^+$ $CD4^+$ T cells before in vitro stimulation for these studies. As shown in Fig 6, both the CD45RA/RO-defined subsets responded to the PMA + I at these later time points with a marked upregulation of CD69 and substantial production of IL-2, yet only the $CD45(RA^-)RO^+$ subset produced significant γ-IFN.

To examine the degree of overlap between the $CD4^+$ memory/effector T-cell populations synthesizing γ-IFN, IL-2, and IL-4 after short-term PMA + I stimulation, we used our flow cytometric procedure to simultaneously analyze pairs of these cytokines among purified memory/effector T cells, with gating on the $CD8^-$ ($CD4^+$) subset (Fig 7). The results indicate a strikingly complex, yet consistent, pattern of cytokine synthesis-defined, $CD4^+$ memory/effector T-cell subsets. In all experiments, the most common memory/effector phenotype was the production of IL-2 alone (30.4% ± 2.0% of cells; n = 5), followed by the IL-2 + γ-IFN-producing phenotype (16.4% ± 2.6%), the γ-IFN alone-producing phenotype (7.7% ± 1.5%), and, finally, the phenotype characterized by IL-4 production (4.3% ± 0.8%), either alone or in combination with other cytokines (see below). Kinetic studies (Fig 8) indicated the IL-2 + γ-IFN–producing subset was relatively short-lived, compared with the IL-2 alone- or γ-IFN alone-producing subsets, practically disappearing by the 22-hour time point. Interestingly, the IL-4– and γ-IFN–producing $CD4^+$ T subsets were predominantly nonoverlapping, whereas, in contrast, the majority of IL-4 producers simultaneously produced IL-2 (Fig 7). These results were confirmed and extended in experiments simultaneously analyzing all three cytokines on PMA + I-stimulated T-cell populations gated on the IL-4–producing subset (Fig 9). A total of 51.1% ± 1.9% (n = 3) of IL-4–producing T cells also produced IL-2; 23.0% ± 4.3% of these T cells produced IL-4 alone, 16.3% ± 3.5% produced IL-4 in combination with both IL-2 + γ-IFN, whereas only 9.6% ± 0.5% produced IL-4 in combination with γ-IFN but not IL-2.

To determine whether the ability of $CD4^+$ memory/effector T cells to heterogeneously produce the cytokines under study was a function of the nature of the activating stimulus, the presence or absence of accessory cells, or the site from which the T cells were obtained, we performed similar multiple intracellular cytokine analyses on superantigen-stimulated $CD4^+/CD45RO^+$ T cells from both PB and from skin blisters overlying cutaneous DTH sites. The PB $CD4^+/CD45RO^+$ T cells were prepurified by negative selection, reconstituted with monocytes, and, after SEA+SEB stimulation, analyzed concomitantly for intracellular cytokine and CD69 expression, with cytokine responses measured on the activated (CD69-gated) population. The skin

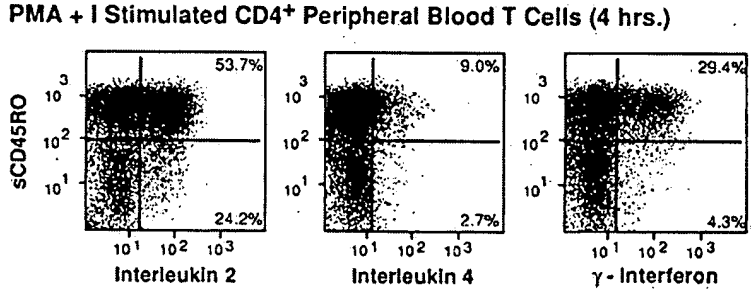

Fig 5. Characterization of the CD45RO phenotype of cytokine-producing $CD4^+$ PB T cells after short-term PMA + I stimulation. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of BFA and then assessed for their correlated expression of cell surface CD45RO versus intracellular cytokine (IL-2, IL-4, γ-IFN) and CD8. Representative profiles (10,000 events are shown in each plot) are shown, gated on the $CD8^-$ ($CD4^+$) subset. The percentages in the upper right and lower right corners of each plot represent the net percentage (%) of positive (+) cells among the $CD45RO^{high}$ and $CD45RO^{low}$ subsets, respectively. As discussed in the Materials and Methods section, BFA prevents activation-induced changes in surface CD45RO (sCD45RO) expression, leaving the sCD45RO expression pattern essentially identical to that observed among fresh T cells (data not shown).

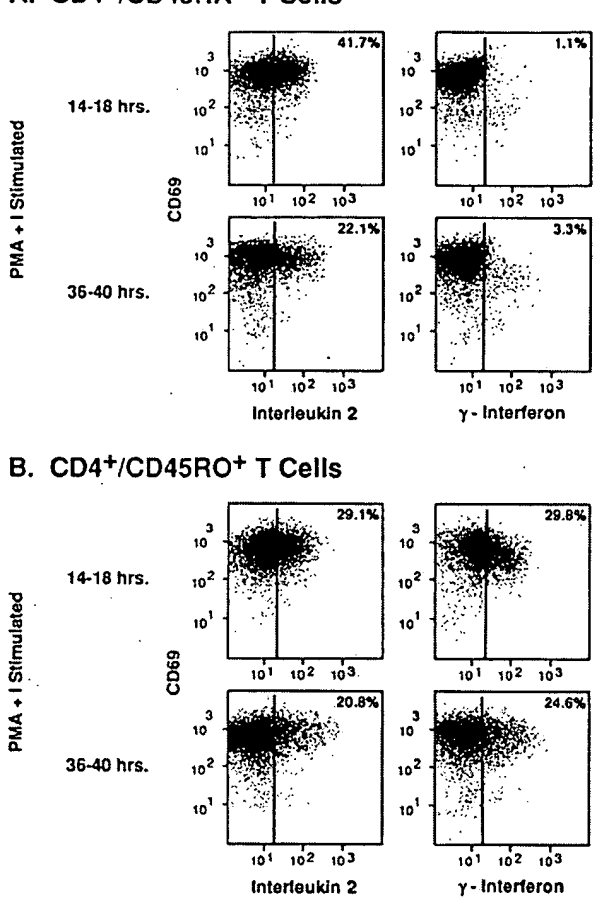

Fig 6. Characterization of the CD45RO phenotype of cytokine-producing CD4+ PB T cells after longer term PMA + I stimulation. CD45RA^{high}/RO^{low} and CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 18 and 40 hours (with BFA included the last 4 hours), and then analyzed for their correlated expression of intracellular cytokine (IL-2 and γ-IFN only; IL-4 is not significantly produced by CD4+ T cells at these time points), CD8, and CD69. Representative profiles of cytokine versus CD69 (5,000 events in each plot) are shown, gated on the CD8$^-$ (CD4$^+$) population. The percentages in the upper right corners of each plot represent the net percentage (%) of cytokine-positive (+) cells among the cells shown. Note the high CD69 expression by all populations at both time points, consistent with nearly universal activation, and the strong production of IL-2 by both the CD45RA^{high}/RO^{low} and CD45RA^{low}/RO^{high} subsets; yet, only the CD45RA^{low}/RO^{high} (CD4$^+$) T cells produce significant γ-IFN.

blister T cells were simultaneously analyzed for two cytokines and CD4, with gating on the CD4+ population. These skin-derived T cells are essentially all CD45RO+, to begin with[1,15] and show an intrinsic twofold to fourfold higher response to SEA + SEB, obviating the need for CD69 gating (data not shown). As shown in Figs 10 and 11, the pattern of cytokine-defined T-cell subsets generated by superantigen stimulation of PB and cutaneous T cells are remarkably similar to each other and to the pattern observed with PMA + I, showing in each circumstance a similar relative proportion of cells synthesizing each of the three studied cytokines, alone and in combination.

DISCUSSION

The concept of cytokine synthesis heterogeneity among memory/effector T-cell populations was initially suggested by data in the mouse indicating that otherwise similar, cloned CD4+ T cells differed in their cytokine production capabilities.[5,8-10] Two general patterns were observed: (1) the TH1 pattern characterized by IL-2, γ-IFN, and tumor necrosis factor β (TNF-β) production; and (2) the TH2 pattern characterized by IL-4, IL-5, IL-6, IL-10 and (later) IL-13 production. The physiologic relevance of these TH1 and TH2 T-cell clones was suggested by the demonstration of polarized TH1- and TH2-like cytokine responses in different in vivo situations that frequently correlated with immune protection against a variety of infectious agents.[9,27,28] More recently, analysis of T-cell clones in the human have shown an analogous, although not identical, cytokine synthesis heterogeneity.[6,7] In addition, bulk cytokine analysis techniques (measurement of secreted cytokines and cytokine mRNA from complex, nonclonal populations), as well as enzyme-linked immunospot assays that allow determination of the number of cells secreting a single cytokine, have indicated that in vivo-derived human T-cell effectors from different disease states or immune situations are often associated with different relative ratios of cytokine gene expression and/or secretion.[29-32]

However, despite the wealth of data on polarized cytokine responses in both mice and humans, methodological limitations have precluded a detailed understanding of the cytokine synthesis potential of single in vivo-derived T cells in either species. The vast majority of studies addressing the ability of individual cells to simultaneously produce two or more cytokines have relied on the study of T-cell clones that have, by necessity, spent weeks to months in vitro before analysis. Although it is unclear the extent to which bias is introduced by this long period of cell culture, it is possible that true in vivo T-cell phenotypes may be modified during T-cell cloning by both selection (only those cells capable of extensive cell division are evaluated) and the inadvertent regulation of cytokine synthesis phenotype. The latter would potentially be mediated by incidental cytokines that may have been added to the culture media to facilitate T-cell outgrowth or produced by the activated T cells themselves and/or their accessory cells during the cloning process. We reasoned that one feasible methodological approach to this problem was multiparameter immunofluorescent analysis of secretion-inhibited, short-term–activated, normal T cells. This technique allows simultaneous assessment of the synthesis of multiple cytokines by T cells that have been cultured in vitro as few as 4 hours. Both the limited time spent in vitro and the secretion inhibition (eg, BFA treatment) greatly diminishes the possibility that T-cell or accessory-cell cytokines could modulate the cytokine response patterns of the T cells. Moreover, because proliferation is not a requirement for a responding cell, and because in most experiments there is little or no cell death during the short-term activation period (L.J. Picker, unpublished observations), selection is unlikely to

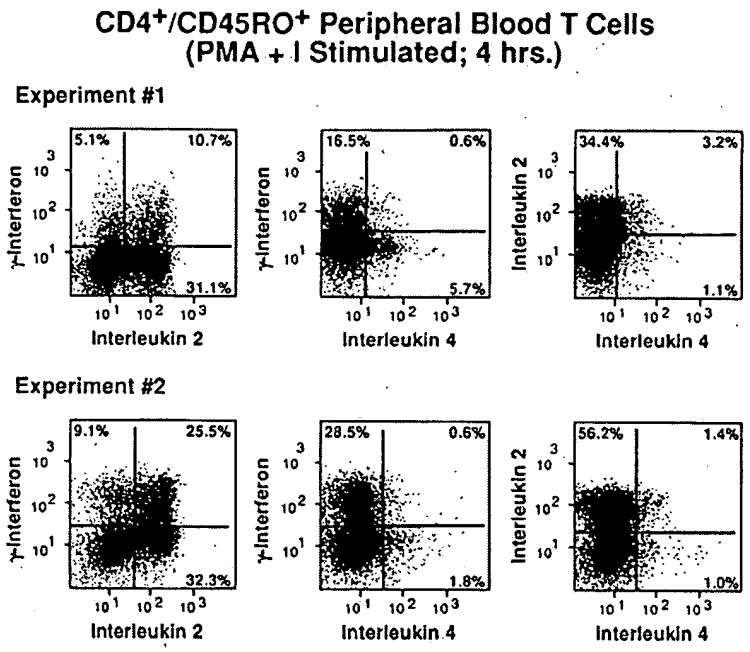

Fig 7. Characterization of patterns of multicytokine production by PMA + I-stimulated CD4⁺/CD45RO^{high} PB T cells. CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 4 hours in the presence of BFA, and then analyzed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2; γ-IFN versus IL-4, and IL-2 versus IL-4) and CD8. Two representative experiments are shown with 10,000 events in each plot, gated on the CD8⁻ (CD4⁺) population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

significantly bias the observed results. Finally, this technique allows simultaneous correlation of cytokine synthesis capabilities with other surface or cytoplasmic phenotypic markers, allowing determination of the cytokine synthesis potential of precisely defined T-cell subsets.

In the first part of this report, we showed the ability of multiparameter flow cytometric cytokine analysis to determine the cytokine synthesis capabilities of the CD4⁺, CD8⁺, CD4⁺/CD45RO^{low}, and CD4⁺/CD45RO^{high} T-cell subsets. Our results indicate that the relative cytokine production capabilities of CD4⁺ and CD8⁺ T cells are in the order of IL-2 > γ-IFN > IL-4 and γ-IFN > IL-2, respectively. In some experiments, we also identified a small IL-4–producing CD8⁺ T-cell subset, consistent with recent reports indicating the existence of such cells.[33,34] Both the absolute and relative percentages of IL-2–, IL-4–, and γ-IFN–producing CD4⁺ versus CD8⁺ T cells found in our study were remarkably similar to the percentages reported by Lewis et al[35] using in situ hybridization techniques. Also consistent with previous reports is our observation that, among CD4⁺ T cells, the CD45RO^{high} putative memory/effector subset accounts for the preponderance of IL-4 and γ-IFN production, whereas both the CD45RO^{high} and CD45RO^{low} virgin subset can produce IL-2.[23,26,36] Both our study and the few others in the literature using techniques capable of measuring cytokine expression/production on a per-cell basis[13,35,38] strongly suggest that these subset-based differences in cytokine production are largely attributable to differences in the frequency of cells capable of producing significant levels of these cytokines, as opposed to differences in either the amount of cytokine produced per cell or the kinetics of production. For example, the higher γ-IFN production by CD4⁺/CD45RO^{high} T cells as compared with that by CD4⁺/CD45RO^{low} T cells[23] was not due to homogeneous high production of γ-IFN by the former versus homogeneous low production by the latter subset, but rather to the ratio of γ-IFN–producing and nonproducing cells in each population. Indeed, for any given T-cell subset (CD4 versus CD8; CD45RO$^{high\ versus\ low}$), the number of IL-2–, γ-IFN–, and IL-4–producing cells generated in response to either PMA + I or superantigen was a consis-

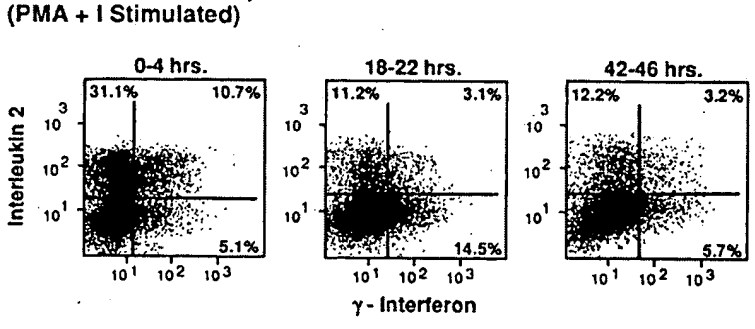

Fig 8. Kinetics of multicytokine-producing (γ-IFN versus IL-2) CD4⁺/CD45RO^{high} PB T cells after PMA + I stimulation. CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, stimulated with PMA+ I for 4, 22, and 46 hours with BFA added during the final 4 hours of culture, and then analyzed for their correlated expression of γ-IFN, IL-2, and CD8. Representative profiles of γ-IFN versus IL-2 (10,000 events in each plot) are shown, gated on the CD8⁻ (CD4⁺) population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

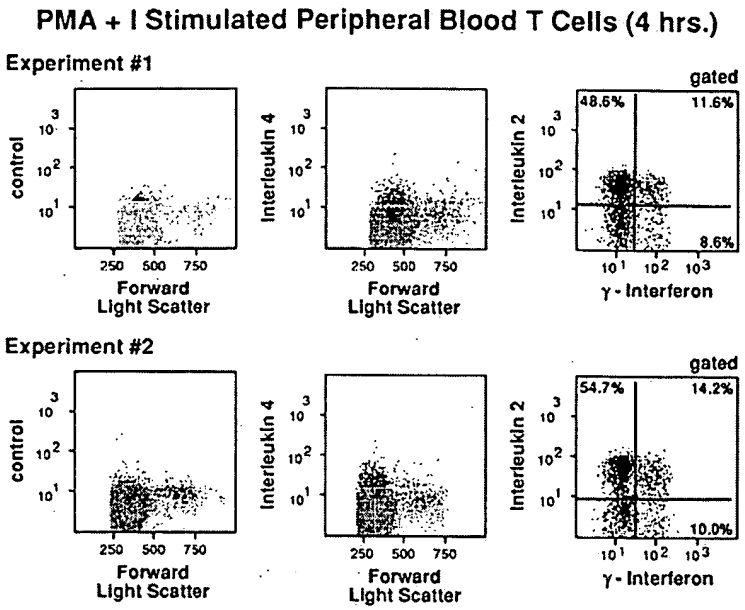

Fig 9. Characterization of γ-IFN and IL-2 production by IL-4 producing PB T cells. Purified PB T cells were stimulated with PMA + I for 4 hours in the presence of BFA and then analyzed for their correlated expression of γ-IFN, IL-2, and IL-4. Two representative experiments are shown. In the left and middle panels (forward light scatter versus IL-4 or isotype-matched control), the overall T-cell subset is shown with the positive cells colored black, and the remaining T cells gray (28,000 to 30,000 events shown). Specific IL-4 staining (after subtracting background) was 1.2% and 1.3% for experiments no. 1 and 2, respectively. The right panels show the γ-IFN versus IL-2 profiles of the IL-4⁺ cells only (2,000 events shown; gated on the black-colored cells in the middle panel). As previously shown (Figs 1 and 5; and confirmed in parallel analyses in these experiments for CD4), under these conditions, a significant IL-4 response is only found in the CD4/CD45RO^{high} T-cell subset, obviating the need for subset prepurification or gating on these parameters in these experiments. The percentages in the corners of the gated plots represent the percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets for IL-2 and γ-IFN, with the −/− population constituting the remaining cells). Note (as previously indicated in the text) that the vast majority of IL-4–producing cells have the light scatter characteristics of small lymphocytes (low forward light scatter), consistent with cytokine synthesis predominantly preceding blastogenesis during the activation response.

tent fraction of the overall activated (CD69⁺) T-cell population. These cytokine-producing T cells manifested characteristic levels of intracellular cytokine, and they appeared and disappeared with predictable kinetics.

The differences in the overall number of CD4⁺/CD45RO^{high} T cells producing IL-2 versus γ-IFN in these initial studies strongly suggested a divergence from the predicted IL-2⁺/γ-IFN⁺ phenotype of TH1 memory/effector T cells. Simultaneous evaluation of two to three cytokines within the same cells confirmed this divergence and showed the existence of distinct subsets of memory/effector T cells that manifested synthesis of all possible combinations of the three cytokines assessed. Most common were cells producing IL-2 alone (30%). Classic TH1 (IL-2⁺/γ-IFN⁺/IL-4⁻) and TH2 (IL-2⁻/γ-IFN⁻/IL-4⁺) patterns were displayed by only a fraction (16% and <1%, respectively) of the PMA + I-activated, memory/effector T cells. Interestingly, the TH1-like IL-2⁺/γ-IFN⁺ subset generated by PMA + I appeared only transiently at the 0- to 4-hour time point; by 18 hours, most of this subset either disappeared or converted to single IL-2– or γ-IFN–producing cells. Among the small, transiently-appearing IL-4–producing subset, 75% also produced IL-2, γ-IFN, or both. Taken together, these data clearly indicate that there is no obligate pairing of the ability to produce these three cytokines during the development of memory/effector T-cell function and strongly suggest that the expression potential of each of these three cytokines is independently regulated. Similar conclusions were drawn by Assenmacher et al[38] who used multiparameter flow cytometry to analyze cytokine expression patterns among murine splenic CD4+ T cells stimulated with SEB. Because cytokine synthesis phenotypes are thought to initially develop during the Ag-induced differentation of virgin to memory/effector T cells in secondary lymphoid tissues,[1,8-10] the myriad cytokine synthesis-defined T-cell subsets observed in this study are consistent with a highly complex spatial and/or temporal regulation of this differentiation process. The relative rarity of IL-4/γ-IFN double-producing memory/effector T cells suggests a relatively nonoverlapping, independent regulation of these two cytokines, as opposed to a likely more promiscuous regulation of IL-2.

We further showed that the pattern of cytokine-defined subsets was qualitatively similar after (1) PMA + I stimulation of accessory cell-depleted PB memory/effector T cells, (2) superantigen stimulation of monocyte-reconstituted PB memory/effector T cells, and (3) superantigen stimulation of skin blister-derived memory/effector T cells that were stimulated in the context of skin-derived accessory cells. Preliminary data also indicate that T-cell stimulation with anti-CD3 plus anti-CD28 yields analogous cytokine-produc-

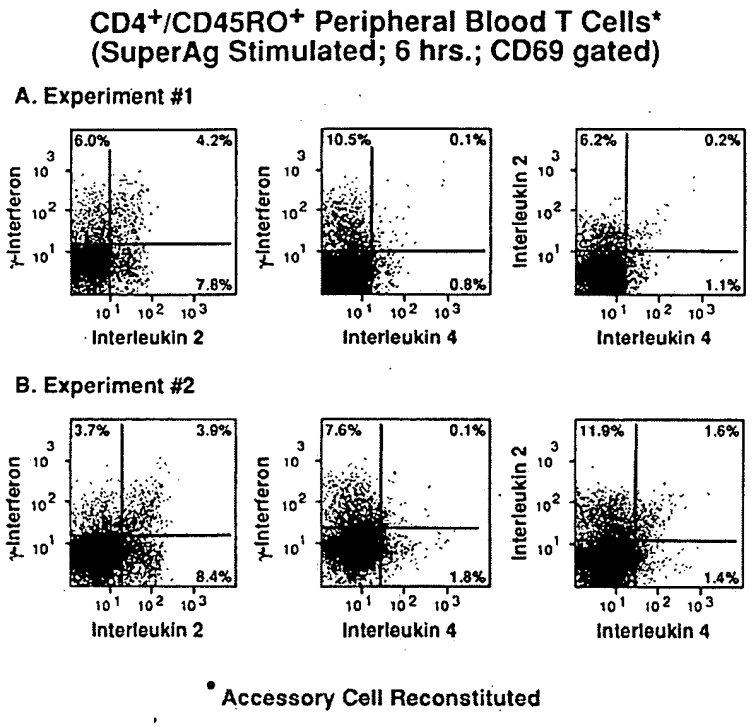

Fig 10. Characterization of patterns of multicytokine production by superantigen-stimulated CD4⁺/CD45RO^{high} PB T cells. CD4⁺/CD45RA^{low}/RO^{high} T cells were prepurified by negative selection, reconstituted with monocytes, and then stimulated with the bacterial superantigens SEA + SEB for 6 hours in the presence of BFA. These cells were then assessed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2, γ-IFN versus IL-4, and IL-2 versus IL-4) and CD69. Two representative experiments are shown with 10,000 events in each plot, gated on the CD69⁺ population, as shown in Fig 2. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

ing subsets (L. Picker and B. Ferguson-Darnell, unpublished observations). These findings strongly suggest that, in the setting of a fully activating stimulus, the observed heterogeneity of memory/effector T-cell cytokine production is an intrinsic characteristic of the T cell and not a function of the regulatory influences of the activating signal, or the activating microenvironment. (The caveat "fully activated" is important because cytokine synthesis-defined memory/effector T-cell subsets have been shown to differ in their activation requirements,[18,39] making it possible that some threshold stimuli may differentially activate a particular subset and not another, thus giving the false appearance of a short-term, functional plasticity among memory/effector T cells.) The stability of this memory/effector T-cell cytokine program-

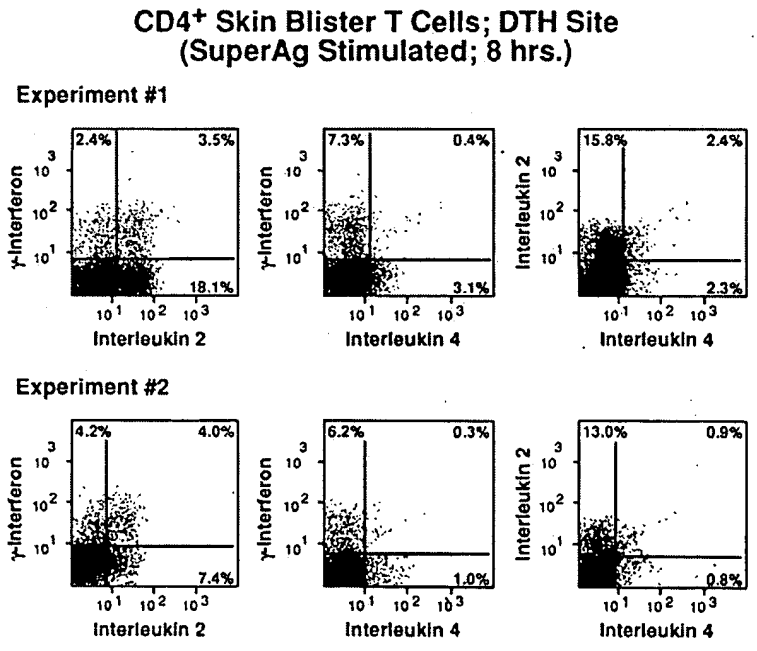

Fig 11. Characterization of patterns of multicytokine production by superantigen-stimulated CD4⁺/CD45RO^{high} cutaneous T cells. Leukocytes obtained from skin blisters overlying DTH reactions (against mumps virus Ag) were stimulated with the bacterial superantigens SEA + SEB for 8 hours in the presence of BFA. These cells were then assessed for their correlated expression of intracellular cytokines (γ-IFN versus IL-2, γ-IFN versus IL-4, and IL-2 versus IL-4) and CD4. Two representative experiments are shown with 10,000 and 5,600 events in each plot in experiments no. 1 and 2, respectively; both gated on the CD4⁺ T cells population. Because of the twofold to threefold increased reactivity to bacterial superantigens manifested by these skin-derived T cells versus that of PB T cells, it was not necessary to use CD69 gating to effectively visualize the cytokine response of this predominantly (>95%) CD45RO^{high} skin T-cell population. The percentages in the corners of each plot represent the net percentage (%) of positive (+) cells in the appropriate quadrants (ie, the +/−, +/+, −/+ subsets).

ming has recently been attested to in experiments showing that the specific cytokine synthesis phenotype of adoptively transferred murine T-cell clones are retained for prolonged periods in vivo.[40]

It is important to note that, although we feel that our data suggest that the ability of memory/effector T cells to synthesize IL-2, IL-4 and γ-IFN is independently preprogrammed, they do not imply that all the cytokine-defined T-cell subsets identified in this report are independent of each other. Because it has been suggested that mouse TH1 and TH2 memory/effector T cells differentiate via TH0 (IL-2$^+$/IL-4$^+$/γ-IFN$^+$) intermediates[5,9] or, in some instances, via intermediates that produce IL-2 alone,[41] it is possible that the analogous human subsets reported here may represent intermediate forms that will terminally differentiate at some future encounter with Ag ± other relevant microenvironmental conditions. However, the presence of these putative memory/effector T-cell intermediates among circulating resting T cells and among T cells in inflammatory sites suggests that they can be fully contributory to T-cell mediated immune responses, and, thus, their putative intermediate status should not be confused with lack of functionality.

The technical simplicity and rapidity of the flow cytometric intracellular cytokine detection technique described in this report, as well as the widespread availability of appropriate flow cytometers and T-cell phenotyping antibodies in clinical laboratories, suggest the possiblity that this technique could be broadly applicable to the clinical evaluation of immune status. For example, previous studies (using more cumbersome immunologic assays) have suggested that alteration of T-cell effector function may be an earlier marker of progressing human immunodeficiency virus-associated immune dysfunction than the absolute loss of whole T-cell subsets (eg, the CD4$^+$ subset).[42] Our observation of qualitatively and quantitatively consistent patterns of memory/effector T-cell cytokine expression in normal subjects offers the possibility that changes in these patterns may precede and predict the clinical manifestations of progressive immunodeficiency. Therefore, flow cytometric quantitation of the cytokine-defined memory/effector T-cell subsets described in this report may prove to be an invaluable aid in both the diagnosis and monitoring of pathologic immunodeficiency and therapeutic immunosuppression.

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Application Note 1

Detection of Intracellular Cytokines in Activated Lymphocytes

Scope

Cytokines are soluble proteins that have a significant role in the immunoregulation of lymphocyte responses.[1,2] Specifically, cytokines regulate the growth, differentiation, and function of a wide variety of cells and mediate normal and pathological immune responses. Cytokines are unique proteins that may have both effector and regulatory activities. In fact, recent research has shown that cytokines can have multiple functions, target many cellular subsets, and can be expressed by diverse cellular subsets.[3,4]

Early studies attempting to relate cytokine expression and T-lymphocyte function were based on activities of cloned cell populations.[5] Although investigations using T-lymphocyte clones demonstrated distinct patterns of cytokine synthesis, ie, T_H1 (IL-2, IFN-γ) and T_H2 (IL-4, IL-5, IL-10), these studies were difficult to interpret because the functional correlation of cloned T lymphocytes to in vivo T-lymphocyte behavior is largely unknown.[6]

Recently, Jung et al[7] and Picker et al[8] have adapted a method to detect the intracellular expression of cytokines after incubation with drugs, such as monensin or Brefeldin A (BFA). This process disrupts intracellular Golgi-mediated transport and allows cytokines to accumulate, yielding an enhanced cytokine signal that can be detected by flow cytometry. This unique method can detect multiple cytokines per cell and discrete cellular populations that express a particular cytokine. These are important advantages when studying cytokine responses to specific stimuli.

At the cellular level, this approach demonstrates the existence of Type 1 and Type 2 polarities in both mouse and human lymphocytes and also shows that these polarities can be reversed under appropriate cytokine-enhanced conditions.[8-10] It is also clear from these studies that only activated cell populations express cytokines; resting normal lymphocytes (T, B, or natural killer [NK]) do not constitutively express cytokines.

The intracellular flow cytometric assay described in this procedure forms the basis of the current BDIS FastImmune™ Cytokine System.* When the anti-cytokine conjugates are used in combination with cell-surface or intracellular phenotyping, the production of cytokines by specific cell types can be assessed.[8] The assay format takes advantage of the unique chemistry and antibody selection protocols BDIS uses to ensure minimal background fluorescence in resting or cytokine-negative cells. Anti-cytokine monoclonal antibody conjugates from BDIS allow detection at low Ig concentrations because they are specifically selected for high-affinity binding to fixed, nascent, intracellular cytokine epitopes.

* For research use only. Not for use in diagnostic or therapeutic procedures.

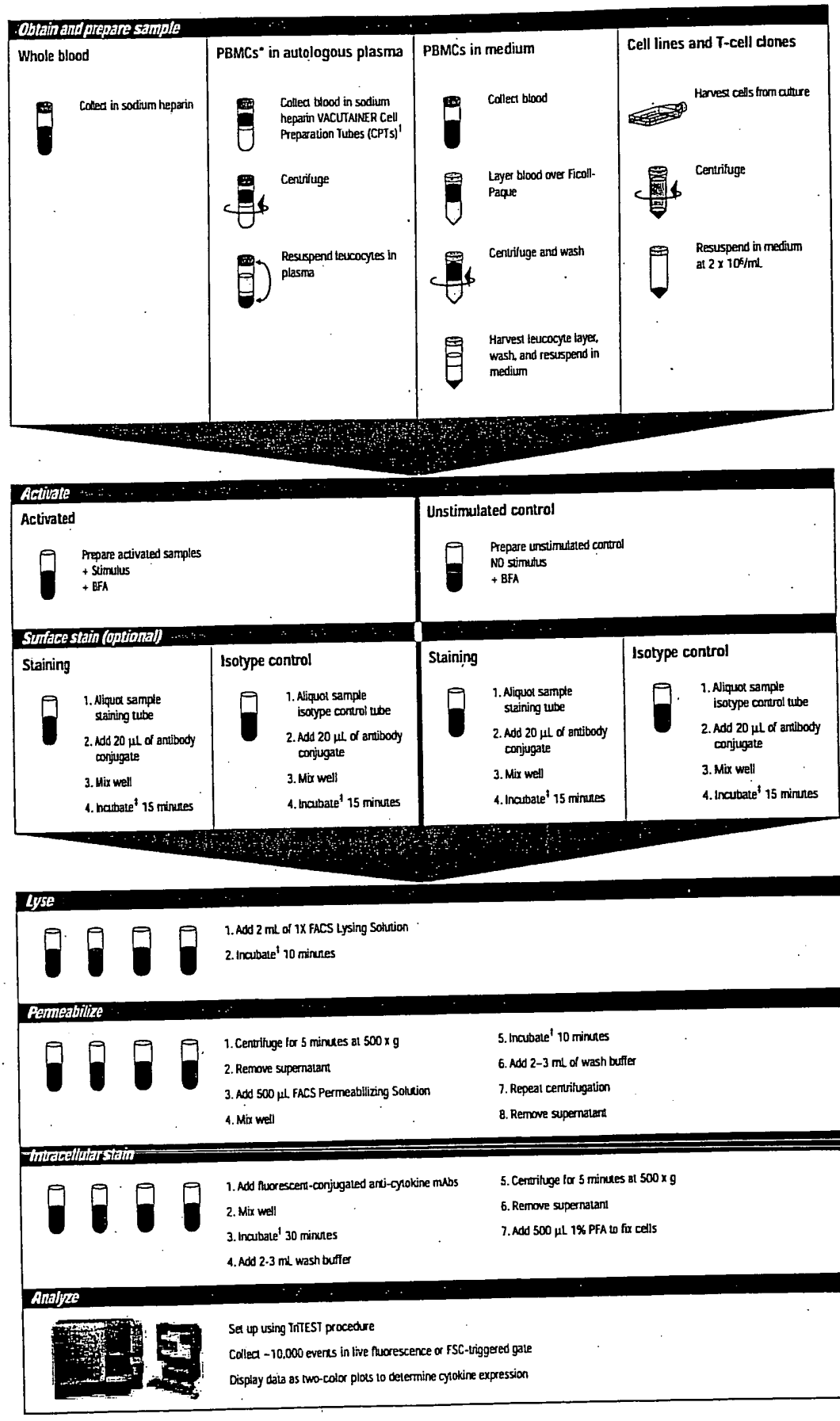

Figure 1 FastImmune intracellular cytokine assay strategy

* Peripheral blood mononuclear cells.

† For more information, contact BD VACUTAINER Division at (800) 631-0174.

‡ Incubate in the dark at room temperature.

Procedure

This procedure is designed for a broad range of applications and with special consideration to minimizing potential assay problems. Figure 1 outlines a general strategy for using this procedure.

Although designed to be simple and rapid, immune function assays require particular attention to the activation steps and reagents, which must be carefully prepared, stored, and used. We include specific recommendations and guidelines for activation, based upon the experiences of the BDIS Immunology Research group.

Successful activation is critical to achieving meaningful results. Therefore, it is important to collect and prepare cells for activation without introducing agents that will interfere with natural activation processes. Avoid calcium-chelating anticoagulants, such as ACD and EDTA, since they restrict calcium-dependent activation responses. On the other hand, lipopolysaccharide (LPS), a common contaminant of biological reagents, is a potent cell activator and can lead to confusing results.

We recommend you begin with experiments that follow our protocol on normal blood samples before using modified procedures or analyzing abnormal samples.

See Troubleshooting at the end of this procedure if you experience difficulty in achieving the desired results.

Equipment

1. Disposable 12 x 75-mm capped polystyrene Falcon® test tubes (Becton Dickinson Labware Catalog No. 2058), or equivalent
2. 37°C incubator with 7% CO_2
3. Vortex mixer
4. Centrifuge
5. Pipetman, or equivalent pipettors
6. FACS® brand flow cytometer

Cells

Whole blood

Collect blood for whole blood activation assays into sodium heparin VACUTAINER® tubes (BD VACUTAINER Catalog No. 367673). FASTIMMUNE assays are incompatible with lithium heparin, EDTA, and ACD anticoagulants.

For best results, assay blood within 8 hours of collection since a minor loss of activity can be expected beyond 8 hours; typically, the percent of cytokine-positive cells is reduced by approximately 5%. If blood cannot be used within 8 hours, store VACUTAINER tubes horizontally at room temperature.

Peripheral blood mononuclear cells (PBMCs) in autologous plasma

Prepare PBMCs using BD VACUTAINER Cell Preparation Tubes (CPTs) (BD VACUTAINER Catalog No. 362753) containing sodium heparin. The VACUTAINER CPT is a blood draw tube with a thixotropic matrix that establishes a density-gradient plug upon centrifugation. See the VACUTAINER CPT product insert for detailed information. Red blood cells (RBCs) and granulocytes pass through the plug to the bottom of the tube while the leucocytes and plasma remain above the plug. By gently inverting the tube, the leucocytes can be conveniently resuspended in the plasma and activated like whole blood in this assay.

Before storage, centrifuge CPTs and resuspend PBMCs in the autologous plasma by gently inverting each tube several times. Store each CPT at room temperature on its side. Assay the blood no later than 24 hours after collection.

PBMCs in tissue culture medium

PBMCs can also be separated via Ficoll-Paque density-gradient centrifugation. Use standard techniques and resuspend at 2 x 10^6 cells/mL in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS) for activation.

Cell lines and T-lymphocyte clones

For activation, resuspend cells at 2 x 10^6 cells/mL in the fresh culture medium typically used to grow the cells.

NOTE: Heat inactivate FBS to denature complement.

Frozen whole blood and PBMCs

Lyse and fix activated whole blood or PBMCs using 1X FACS™ Lysing Solution; wash with phosphate-buffered saline (PBS) and freeze in PBS with 1% bovine serum albumin (BSA) and 10% DMSO at –70°C. After thawing, aliquot cells into staining tubes. Wash cells by adding 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*; then permeabilize with FACS Permeabilizing Solution and stain.

Selecting, Preparing, and Storing Reagents

The following procedures and reagents have been successfully used by the research laboratories at BDIS.

Reagents used in activation (not provided by BDIS)

1. Phorbol 12-Myristate 13 Acetate (PMA) (Sigma Catalog No. P-8139)
   a. Reconstitute in DMSO at 0.1 mg/mL.
   b. Store small aliquots (eg, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:100 in sterile PBS (without sodium azide) for each experiment.
   d. Use PMA at a final concentration of 25 ng/mL of cell suspension. (See Activation section in this procedure for additional information on using this reagent.)
2. Ionomycin (Sigma Catalog No. I-0634)
   a. Reconstitute in EtOH at 0.5 mg/mL.
   b. Store at –20°C.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each experiment.
   d. Use ionomycin at a final concentration of 1 µg/mL of cell suspension.
3. Staphylococcal enterotoxin B (SEB) (Sigma Catalog No. S-4881)
   a. Reconstitute in sterile PBS (without sodium azide) at 0.5 mg/mL.
   b. Store at 4°C.
   c. Use SEB at a final concentration of 10 µg/mL of cell suspension. (See Activation section in this procedure for additional information on using this reagent.)
4. Brefeldin-A (BFA) (Sigma Catalog No. B-7651)
   a. Reconstitute in DMSO at 5 mg/mL.
   b. Store small aliquots (eg, 20 µL) at –20°C; do not refreeze aliquots after thawing.
   c. Dilute stock 1:10 in sterile PBS (without sodium azide) for each assay.
   d. Use BFA at 10 µg/mL of cell suspension for the last 4 to 5 hours of activation.

   NOTE: Extensive incubation with BFA will reduce cell viability. (See Activation section in this procedure for additional information on using this reagent.)
5. RPMI-1640 (BioWhittaker Catalog No. 12-167F)
6. PBS without sodium azide (NaN_3), sterile filtered
7. DMSO (Sigma Catalog No. D-8779)
8. EtOH, Gold Shield Ethyl Alcohol, 200 proof
9. Wash buffer, PBS with 0.5% BSA and 0.1% NaN_3; store at 4°C.
10. 1% paraformaldehyde (PFA) in PBS; store at 4°C.

Reagents for immunophenotypic staining (BDIS reagents)

11. Monoclonal antibody conjugates for surface staining

    The choice of specific surface markers will depend on your particular phenotyping strategy. For example, CD45 PerCP (BDIS Catalog No. 347464) is useful for triggering on all lymphocytes. CD3 PerCP (BDIS Catalog No. 347344) is an excellent choice for fluorescent triggering on $CD3^+$ T lymphocytes in a multicolor assay. CD4 PerCP (BDIS Catalog No. 347324) or CD8 PerCP (BDIS Catalog No. 347314) are good choices for triggering on specific T-lymphocyte subsets. CD19 PerCP (BDIS Catalog No. 347544) or CD20 PerCP (BDIS Catalog No. 347674) can be used to identify B lymphocytes, while CD56 and CD14 are the markers of choice for NK lymphocytes and monocytes, respectively.
12. FACS Lysing Solution

    FACS Lysing Solution (BDIS Catalog No. 349202) is required when using PBMC preparations, cultured cells, and whole blood. For this method, it serves three purposes:

    a. lyses RBCs in whole blood preparations
    b. fixes the external epitopes
    c. assists in permeabilization

    FACS Lysing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.

13. FACS Permeabilizing Solution

BDIS has developed FACS Permeabilizing Solution (BDIS Catalog No. 340457) to ensure consistent sensitivity and low background staining. This proprietary reagent is specifically designed to overcome the limitations of saponin-based permeabilizing reagents common to many intracellular staining protocols. Saponin, a compound derived from plants, is a common source of variability in intracellular immunophenotypic staining because of its heterogeneous composition. FACS Permeabilizing Solution also eliminates the necessity of freezing cells overnight, which some protocols recommend to increase permeability.

FACS Permeabilizing Solution is supplied as a 10X concentrate. Before use, dilute 1:10 in deionized water; refer to the product insert for instructions. Do not dilute in PBS or other buffers.

14. Monoclonal antibody conjugates for intracellular staining

The choice of specific intracellular markers will depend on your particular phenotyping strategy. For example, the two-color IFN-γ FITC/IL-4 PE reagent (BDIS Catalog No. 340456) is a popular choice for simultaneously assessing Type 1 and Type 2 immune responses with fluorescent triggering on CD3 PerCP or other markers.

Successful intracellular staining demands superior antibodies against the intracellular targets. Intracellular assays that utilize secretory inhibitors, such as BFA, detect developing protein in the Golgi apparatus. Conformationally, the nascent protein may be different from the secreted form. Therefore, antibodies that work well in detecting secreted cytokine may perform poorly in intracellular assays. BDIS screens many monoclonal antibodies for each cytokine under actual intracellular assay conditions.

Activation

Activation is performed in the presence of BFA which inhibits intracellular transport of proteins,[8,9,11] so antigens and cytokines produced during activation will be retained inside the cell. The unstimulated control sample should also contain BFA. See the Assay Control section in this procedure. All activation procedures outlined are performed in 12 x 75-mm capped polystyrene test tubes (Falcon Catalog No. 2058). The reagent concentrations indicated are final concentrations in the activation mixture using reagent preparations described previously.

1. PMA + ionomycin (PMA + I)
   a. Dilute whole blood or PBMCs in plasma 1:1 with RPMI 1640 without serum. (This dilution procedure is required only for PMA + I activation. Cells that have already been resuspended at 2 x 10^6/mL in medium need not be further diluted with RPMI.)
   b. Stimulate with 25 ng/mL of PMA (25 µL of working solution described previously per mL of blood) and 1 µg/mL of ionomycin (20 µL of working solution per mL of blood) in the presence of 10 µg/mL of BFA (20 µL of working solution per mL of blood).
   c. Incubate for 4 hours at 37°C, 7% CO_2 with tube caps loosened to allow entry of CO_2-containing air. (While a CO_2 incubator is preferred to ensure proper control of pH, the incubation can also be carried out in a water bath with each tube tightly capped.)
2. SEB
   a. Activate undiluted blood with 10 µg/mL of SEB in the presence of 10 µg/mL of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
3. CD2/CD2R (BDIS Catalog No. 340366)
   a. Activate undiluted blood with 20 µL of CD2/CD2R per mL of blood in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C.
4. CD3
   a. Activate undiluted blood with immobilized CD3[12] in the presence of BFA.
   b. Incubate for 4 to 6 hours at 37°C. CD5 PerCP (BDIS Custom Conjugate) or CD45 PerCP (BDIS Catalog No. 347464) are recommended for FL3 fluorescence triggering because the CD3 antigen is modulated by crosslinking of CD3.

   NOTE: High-concentration, low-azide CD3 is available through the BDIS Custom Conjugate Program. Contact your local BDIS representative for more information.

5. CD28

Use CD28 (BDIS Catalog No. 550017) at 10 µg/mL to enhance activation responses to various stimuli, including SEB, CD3, and CD2/CD2R.

Assay Controls

Proper system controls simplify troubleshooting. Before modifying the procedure, it is strongly recommended that you establish proficiency with the assay by using normal human samples described in this procedure. At the start, prepare all of the controls outlined below using normal human donor samples. As you gain confidence in your results, you can eliminate the activation and intracellular staining controls. Figure 4 in the Troubleshooting section illustrates how the activation and intracellular staining controls are useful in troubleshooting. However, you should run unstimulated and isotype controls for each sample in the assay.

1. Unstimulated control

The unstimulated control is used to assess the level of residual cytokine synthesis from in vivo activation. Run this control for all samples. As the name implies, the unstimulated control is prepared by incubating the blood during the activation step with 10 µg/mL of BFA, but without a stimulus.

2. Isotype controls

Fluorescent-conjugated isotype control antibodies are used at matching concentrations to detect non-specific binding to cells due to the class of the mouse monoclonal antibody. The FastImmune Cytokine System utilizes standard anti-KLH isotype controls specially formulated for intracellular detection systems. These intuitive controls save time and resources by eliminating the need for expensive and tedious cross competition with unlabeled recombinant cytokines.

3. Activation control

The activation control utilizes surface expression of CD69 to assess whether activation has been achieved. If the expected level of CD69 is not seen, there is a problem with the activation step of the assay. Specifically, one of the reagents used in the activation step may be inactive, expired, or improperly prepared; or, a solvent may be contaminated. Make fresh preparations of the stimuli and try again.

a. Activate one aliquot of blood with PMA + I (as described previously) but omit the BFA.

NOTE: Anti-secretory agents like BFA prevent surface expression of CD69 and must be omitted to permit surface expression and detection.

b. Surface stain only with CD69 PE/CD3 PerCP (BDIS Catalog No. 340368). Omit the permeabilization and intracellular staining steps of the procedure.

c. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Surface staining for CD69 should be greater than 90% positive (Figure 2).

4. Intracellular staining control

The intracellular staining control assesses intracellular staining of CD69 in conjunction with the results of the activation control to pinpoint whether permeabilization and intracellular staining are executed properly. If the activation control is greater than 90% positive but a comparable level of CD69 is not detected by intracellular staining, there is a problem with the permeabilization or intracellular staining step of the assay. Make sure you have

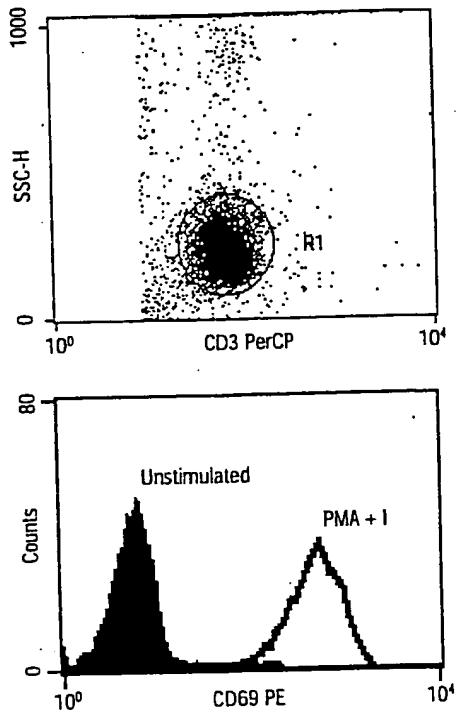

Figure 2 Activation control

followed the procedure exactly as written, and try again.

a. Activate one aliquot of blood with PMA and ionomycin in the presence of BFA.

b. Omit the surface staining step. Stain intracellularly only with CD69 PE/CD3 PerCP (BDIS Catalog No. 340368).

c. Analyze results by fluorescence triggering on FL3 and assessing CD69 staining in the CD3 gated events. Intracellular staining for CD69 should be greater than 90% positive (Figure 3).

Staining

Anti-cytokine antibodies can be used in conjunction with antibodies against cell surface antigens for the purpose of studying specific lymphocyte populations. CD3 PerCP, CD4 FITC, and CD8 FITC are most commonly used by the BDIS research laboratories for T-lymphocyte subsetting. The CD4, CD14, and CD56 antigens are modulated by PMA activation to varying degrees in most donors; therefore, they are not useful for phenotypic subsetting in PMA-activated samples.

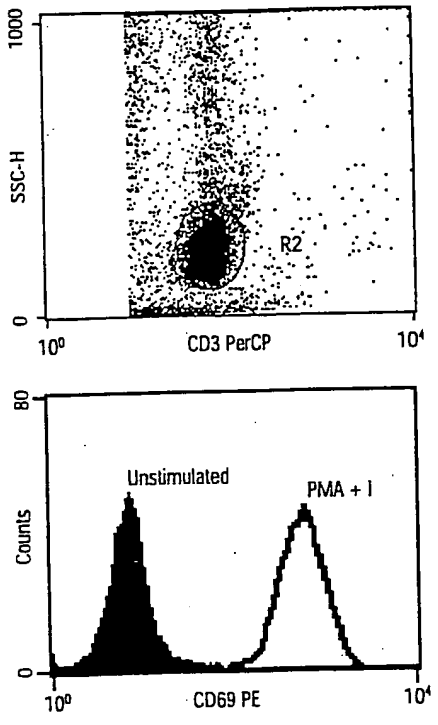

Figure 3 Intracellular staining control

To simultaneously stain all epitopes after permeabilization, add all antibody conjugates at the same time. Surface staining reagents should be titrated if used in the intracellular staining step to ensure optimal results. Intracellular staining of all surface markers should be compared to surface staining to ensure that they are comparable. Staining of CD3, CD4, and CD8 antigens can be performed either on the cell surface prior to permeabilization, or during the intracellular staining step after permeabilization. This does not apply to all antigens because epitopes vary in their sensitivity to paraformaldehyde fixation.

Surface staining

1. Add 20 µL of each BDIS surface staining reagent to 12 x 75-mm tubes.
2. Add 100 µL of diluted PMA + I activated blood or 50 µL of undiluted whole blood (activated by other stimuli) to the surface staining reagents. (Dilution of whole blood or PBMCs in plasma in RPMI is only required for PMA + I activation. Cells which have already been resuspended at 2 x 10^6 cells/mL in medium need not be further diluted with RPMI.)
3. Mix well and incubate for 15 minutes at room temperature in the dark.

Permeabilization and intracellular staining

1. Add 2 mL of 1X FACS Lysing Solution prepared according to the package insert. Incubate for 10 minutes at room temperature. When staining PBMCs or cultured cells, add FACS Lysing Solution to fix the surface epitopes and optimize the permeabilization process.

   NOTE: PMA-activated whole blood does not always lyse completely.

2. Centrifuge for 5 minutes at 500 x *g* and remove the supernatant. Avoid disturbing the pellet. Add 500 µL of 1X FACS Permeabilizing Solution prepared according to the package insert and mix well. Incubate for 10 minutes at room temperature in the dark.
3. Add 2 to 3 mL of wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove the supernatant.
4. Add fluorescent-conjugated anti-cytokine mAbs. Mix well and incubate for 30 minutes at room temperature in the dark.

5. Add 2 to 3 mL wash buffer and centrifuge for 5 minutes at 500 x *g*. Remove supernatant and add 500 µL 1% PFA.

   NOTE: Samples can be stored for up to 24 hours at 4°C in the dark prior to analysis.

Analysis

1. Analyze on a FACS brand flow cytometer.
2. Use CaliBRITE™ beads and appropriate software (FACSComp™, version 1.1 or later, or AutoCOMP™, version 3.0.2) for setting photomultiplier tube (PMT) voltages and fluorescence compensation and for checking instrument sensitivity prior to use. Refer to the appropriate TriTEST™ three-color application note for flow cytometric setup, acquisition, and analysis.

   NOTE: Proper instrument setup with the correct version of FACSComp or AutoCOMP is important for obtaining accurate results with the FastImmune assay. Contact your BDIS representative if you have an older version of either FACSComp or AutoCOMP.
3. Analyze samples on a FACS brand flow cytometric analyzer. Acquire data with CellQuest™ or LYSYS™ II software, using a fluorescence or forward scatter (FSC) threshold. Typically, 10,000 gated events is sufficient.
4. Gate on $FL3^+$ cells. Display data as two-color dot plots to determine cytokine expression. Data may be analyzed using CellQuest, LYSYS II, PAINT-A-GATE™, or Attractors™ software. With PMA activation, platelets can move into the $FL3^+$ gate. In this case, gate on FSC/SSC. In assays with a CD4 trigger, gate on FSC/SSC to exclude monocytes.

Calculate the specific response

As illustrated by the following formula, the specific response of cells to any stimulus is obtained by subtracting % positive events in the isotype control sample from % positive events in the anti-cytokine antibody-stained sample. Then subtract the isotype-corrected response of the unstimulated sample from that of the stimulated sample.

Formula: (AS – AIC) – (US – UIC)

where AS = activated sample
AIC = activated isotype control
US = unstimulated sample
UIC = unstimulated isotype control

Troubleshooting

The following troubleshooting matrix should help you pinpoint potential sources of problems in the assay. The most common sources are the sample preparation and activation steps. The troubleshooting strategy in Figure 4 illustrates how the activation and intracellular staining controls can be used to identify activation problems.

Problem	Possible Cause	Solution	Comments
No intracellular staining of cytokines or CD69	Cells not activated	Activation reagents not prepared correctly. See the Procedure section for stimulus preparation and storage.	>90% $CD3^+$ T lymphocytes should be $CD69^+$ after 4 hour–PMA + I activation.
	Wrong anticoagulant used for blood collection	Use only sodium heparin anticoagulant in blood collection. Do not use lithium heparin. Do not use ACD, EDTA, or other calcium-chelating anticoagulants.	Calcium is required for lymphocyte activation; calcium-chelating anticoagulants prevent activation.
	Cells not permeabilized	Treat cells with FACS Lysing Solution prior to treatment with FACS Permeabilizing Solution.	FACS Lysing Solution conditions cells for permeabilization.
	BFA inactive or prepared incorrectly	Prepare BFA as directed. Aliquot BFA stocks and store at –20°C.	See the Procedure section for preparation of non-BDIS reagents.
Intracellular staining positive but dim	Wrong concentration of anti-cytokine monoclonal antibodies	Use only BDIS directly conjugated monoclonal antibodies at recommended concentrations.	IL-4 is often expressed at frequencies below 2% in normal activated T lymphocytes.
	Permeabilized cells not washed prior to intracellular staining	Wash permeabilized cells as per protocol prior to staining.	
Background staining too high	Poor conjugate purification or antibody conjugate breakdown, resulting in free fluorochrome which binds nonspecifically	Use BDIS FastImmune reagents.	BDIS reagents are carefully designed to minimize background staining. BDIS PE-conjugated isotype controls for intracellular staining are specially formulated for this application. The surface staining isotype controls are formulated differently and may yield high background in intracellular staining applications.
	Antibodies with low affinity for fixed, nascent intracellular antigens requiring high antibody concentrations	Use BDIS FastImmune reagents.	
	Wrong isotype control; Control Ig used at too high concentration	Use BDIS–matched isotype controls for intracellular staining at recommended concentration (matching the concentration of the test antibody conjugate).	
Unacceptable cell loss during procedure	Cells not recovered during centrifuge washing steps	Centrifuge fixed and permeabilized cells at 500 x *g*.	Fixed cells have lower density than live cells; therefore, they require higher centrifugal force to pellet.
	Cells not recovered during aspiration steps	Decant supernatant instead of aspirating with vacuum.	
Incomplete RBC lysis	PMA + I activation	Follow procedures for FL3 triggering to eliminate debris and unlysed cells from analysis.	PMA + I–activated whole blood samples can be difficult to lyse. PMA tends to stabilize RBC plasma membranes.
	FACS Permeabilization Solution or FACS Lysing Solution not diluted 1:10 in deionized (DI) water	Follow protocol for dilution of FACS Permeabilization Solution or FACS Lysing Solution.	These reagents must be diluted in DI water; diluting these reagents in PBS reduces the impact of osmotic differential. Procedure may not be compatible with other permeabilizing reagents.
	Lysis not carried out at room temperature	Lyse at room temperature.	

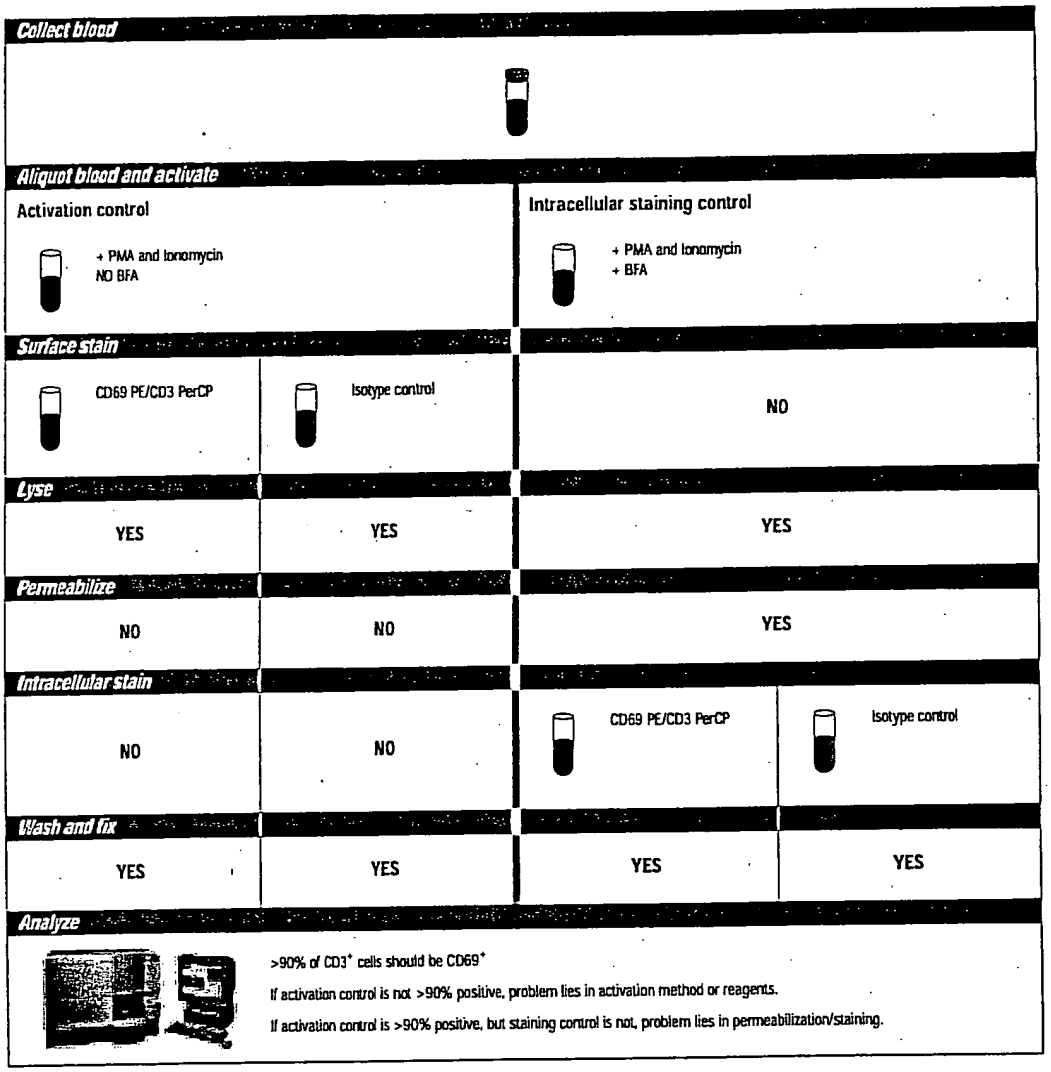

Figure 4 FastImmune cytokine troubleshooting strategy

BDIS publishes this method as a service to investigators. Detailed support for non-flow cytometric aspects of this procedure may not be available from BDIS.

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Patents

FACS Lysing Solution: US Patent Nos. 4,654,312 and 4,902,613.
Phycoerythrin (PE) Conjugates: US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942.
Peridinin Chlorophyll Protein (PerCP): US Patent No. 4,876,190.

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Detection of antigen-specific T cell cytokine expression in whole blood by flow cytometry

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Abstract

We have recently described a highly sensitive flow cytometric technique, based on the ability to detect single cell expression of cytokines, to simultaneously quantitate and phenotypically characterize antigen-specific memory/effector T cells in PBMC cultures. In this report, we describe a simplified procedural modification which enables the rapid detection of low frequency memory CD4 + and CD8 + T cells expressing cytokines in response to soluble antigen in whole blood. When compared with T cell responses in PBMC cultures, whole blood cultures demonstrated similar but slightly higher percentages of T cells responsive to specific antigen. In addition, T cell responses to cytomegalovirus in whole blood were observed only in sensitized (seropositive) individuals, and CD4 + T cell responses could be blocked by anti-class II MHC antibodies. This procedure may provide a means to examine direct effects of pharmacological drug concentrations on T cell immunity in clinical samples. © 1998 Elsevier Science B.V.

Keywords: Flow cytometry; Antigen-specific activation; Cytokine production; Cytomegalovirus; Whole blood activation assay

1. Introduction

Cytokines are polypeptide hormones involved in the regulation of growth, differentiation and function of hematopoietic and non-hematopoietic cells (Street and Mosmann, 1991; Paul and Seder, 1994; Aggarwal and Puri, 1995). Cytokine expression in mature T cells in response to foreign antigen is a primary defense mechanism which characterizes cellular immunity and can influence both normal and pathological immune responses (Rocken et al., 1992; Hillman and Haas, 1995; Openshaw et al., 1995; Zhang et al., 1995; Telander and Mueller, 1997). Cytokine expression patterns can be used, for example, to distinguish normal and abnormal T cell function in a variety of conditions (Clerici et al., 1993; Orme et al., 1993; Elson et al., 1995; Picker et al., 1995; Maino et al., 1996; Garcia et al., 1997). The ability to evaluate these cytokine patterns at the cellular level is critical for obtaining precise information about both the frequencies and the phenotypes of T cells responding to specific stimuli (Jung et al., 1993; Picker et al.,

Abbreviations: Ag, antigen; CMV, cytomegalovirus; BFA, Brefeldin A; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; SEB, staphylococcal enterotoxin B; KLH, keyhole limpet hemocyanin

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1995; Prussin and Metcalfe, 1995; Maino et al., 1996; Prussin, 1996).

We have recently described methods for multiparameter flow cytometric analysis of the expression of multiple cytokines in individual T cells in response to polyclonal stimuli (Picker et al., 1995; Maino et al., 1996) or specific antigen (Waldrop et al., 1997). The assay format demonstrated an accumulation of interleukins 2 and 4 (IL-2 and IL-4), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) in the cytoplasm of CD4 + T cells after a short (4–8 h) incubation with an activating stimulus in the presence of the secretion inhibitor Brefeldin A (BFA; Klausner et al., 1992; Picker et al., 1995). These earlier studies illustrate the capability of this flow cytometric technique to simultaneously quantitate antigen-specific memory/effector T cells in human blood and characterize them functionally and phenotypically.

The ability to evaluate antigen-specific responses in whole blood offers some unique advantages in a number of clinical applications (Maino et al., 1995b; Petrovsky and Harrison, 1995). These include the assessment of the in vivo effects of drug therapies and immune therapies, the measurement of vaccine efficacy, and the assessment of in vitro drug effects on the ability of T cells to mount a specific response to antigen. Specific responses can be measured in the presence of autologous cellular and serum components which may be physiologically relevant.

In this report, we describe a simplified method for measuring antigen-specific T cell responses in whole blood, and demonstrate that similar frequencies of T cells respond to antigen in both whole blood and purified peripheral blood mononuclear cell (PBMC) cultures. We further demonstrate that this method can be used to assess antigen-specific responses in both CD4 + and CD8 + T cell subsets.

2. Materials and methods

2.1. Antigens and antibodies

Cytomegalovirus (CMV) antigen (Ag) was used either as a partially purified virally infected cell lysate (BioWhittaker, Walkersville, MD) or as purified virus (Advanced Biotechnologies, Columbia, MD). The matched CMV control Ag for the partially purified CMV preparation was obtained from BioWhittaker. The control Ag, prepared from non-infected tissue, was utilized in these assays to detect non-specific activation responses. The superantigen staphylococcal enterotoxin B (SEB) was purchased from Sigma Chemical (St. Louis, MO). All antigens were used at optimal stimulatory concentrations as determined by titration experiments.

Monoclonal antibodies (mAbs) CD3 PerCP, CD4 PerCP, CD8 FITC, CD69 PE, anti-IL-2 FITC and PE, anti-IFNγ FITC and PE, anti-TNFα FITC and PE, IgG1 FITC and PE, CD28, anti-HLA-DR (clone L203), anti-HLA-DP, anti-HLA-DQ and CD45 were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA) and used at the manufacturer's recommended concentrations.

2.2. Cell preparation and antigenic stimulation

Sodium heparinized venous blood was aliquoted into 15 ml conical polypropylene tubes (cat# 2096, Becton Dickinson Labware, Franklin Lakes, NJ) at 1 ml per tube. The co-stimulatory mAb CD28 was added to whole blood samples at 3 μg/ml and antigens were subsequently added at optimal stimulatory concentrations (CMV Ag from Advanced Biotechnologies at a final concentration of 2 μg/ml, CMV Ag and control Ag from BioWhittaker at 60 μl/ml). The culture tubes were incubated upright in a humidified 37°C, 7% CO_2 incubator for a total of 6 h, with the first hour of incubation in the absence of BFA to enable antigen processing by antigen presenting cells (APC) (Openshaw et al., 1995; Waldrop et al., 1997). The secretion inhibitor BFA (10 μg/ml, Sigma) was included for the final 5 h of activation (Lippincott-Schwartz et al., 1989; Klausner et al., 1992; Picker et al., 1995). At 6 h, 100 μl of 20 mM EDTA (for a final concentration of 2 mM EDTA) was added directly to the whole blood cultures. Sample tubes were vortexed vigorously and incubated for 15 min at 22°C (RT). Blood samples were then lysed and fixed in 14 ml of 1 × FACS™ Lysing Solution (BDIS) for 10 min at RT. Cells were washed in PBS and frozen at −70°C in a freezing medium containing 10% DMSO and 1% BSA in PBS. Alternatively, cells were resuspended in PBS and aliquoted

into staining tubes for permeabilization as described in Section 2.3. For some experiments, PBMC were isolated using Vacutainer® Cell Preparation Tubes (CPT) containing sodium heparin anticoagulant (Becton Dickinson VACUTAINER Systems, Franklin Lakes, NJ). PBMC were resuspended to the original volume in autologous plasma and activated as described by Waldrop et al. (1997).

2.3. Immunofluorescent staining

Frozen cells were thawed rapidly in a 37°C water bath, aliquoted into staining tubes at approximately 5×10^5 cells per tube and washed once with cold wash buffer (PBS, 0.5% BSA, 0.1% NaN_3). Cells were subsequently resuspended in 0.5 ml of FACS™ Permeabilizing Solution (BDIS) for 10 min at RT. After permeabilization, cells were washed once and staining was performed for 30 min at RT in the dark using a titrated mixture of fluorescent conjugated mAbs. For a typical three-color analysis of a CD4 + cytokine response, the staining antibody cocktail consisted of CD4 PerCP, CD69 PE and anti-IL-2, anti-IFNγ or anti-TNFα FITC. Isotype matched control antibodies were included to detect non-specific binding to cells. After staining, samples were washed and fixed in 1% paraformaldehyde in PBS and stored at 4°C until FACS analysis.

2.4. Flow cytometric analysis

Three-color flow cytometric analysis was performed on a FACScan™ or a FACSCalibur™ flow cytometer (BDIS). Data were acquired using CELLQuest™ software (BDIS), typically collecting 40,000–50,000 gated CD4 + events using FL3 (PerCP) as a fluorescent trigger. Data were displayed as two-color dot plots (FL1 vs. FL2) in PAINT-A-GATE^{Plus} software (BDIS) to measure the proportion of the double-positive (cytokine + /CD69 +) cells. Since all specific cytokine expression occurs within the CD69 + (activated) cell subset, CD69 staining was included to enhance the identification of Ag-responsive T cells, as reported by Waldrop et al. (1997). Forward scatter vs. side scatter gating was employed in data analysis to exclude any CD4 + monocytes.

3. Results

3.1. Cytokine responses to purified CMV in CMV seropositive and seronegative donors

We have previously demonstrated host specificity of Ag-specific T cell responses using a crude preparation of CMV-infected cell lysate (Waldrop et al., 1997). In this report, we show similar host specificity in whole blood cultures using sucrose density gradient purified CMV preparations at low protein concentrations. For these experiments, whole blood from a CMV seropositive donor and a CMV seronegative donor was stimulated with purified CMV virus (Advanced Biotechnologies) as described in Section 2. Fig. 1 shows the cytokine profiles (IL-2, IFNγ and TNFα) of CD4 + /CD69 + T cells in these two types of individuals. Significant frequencies of T cells from the CMV seropositive donor exhibited positive cytokine/CD69 responses to purified CMV (plots A–C), while no cytokine expression was observed in the unstimulated control cultures (plots D–F). Importantly, CD4 + T cells from the seronegative donor failed to exhibit specific cytokine production in response to CMV as detected with any of the anti-cytokine mAbs (plots G–L). In the same assay, roughly equivalent IL-2 and TNFα responses to SEB (10 μg/ml) + CD28 (3 μg/ml) were observed in both the seropositive and seronegative blood samples, demonstrating that the seronegative donor cells were capable of producing these cytokines in response to an appropriate stimulus (data not shown). CD28 alone had no effect on cytokine production.

3.2. CD8 + T cell cytokine responses to CMV Ag

Some investigators have demonstrated CD8 + T cell responses to exogenous Ag by non-cytometric methods (Carbone and Bevan, 1990; Kovacsovics-Bankowski et al., 1993; Germain, 1994; Harding and Song, 1994; Reimann et al., 1994; Reise Sousa and Germain, 1995). Using the method described in this report, we examined CMV Ag-induced cytokine production of CD8 + T cells in whole blood samples

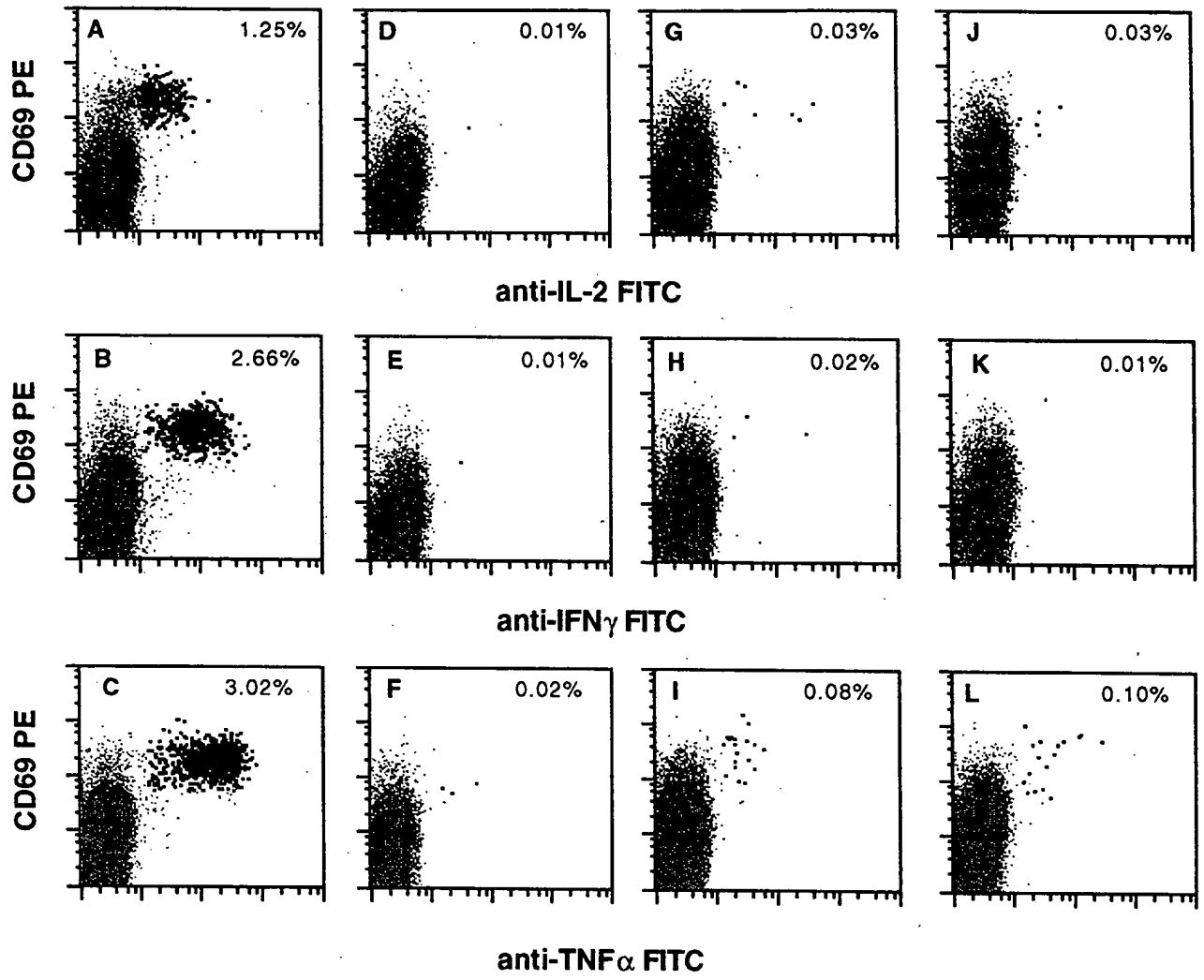

Fig. 1. Purified CMV Ag-induced cytokine responses in a CMV seropositive (plots A–F) and seronegative (plots G–L) donor. Whole blood was stimulated with CMV Ag for 6 h as described in Section 2. Activated cells were stained with CD4 PerCP, CD69 PE and anti-IL-2, anti-IFNγ or anti-TNFα FITC, and 40,000 CD4 + events were analysed for the upregulation of intracellular cytokines and CD69. The enlarged red dots represent the double-positive events (cytokine + /CD69 +), with the percentages displayed in the upper right corner of the plots. Plots A–C depict the CMV-specific response, and plots D–F the response in the unstimulated control culture (which contains 3 μg/ml CD28) of the seropositive subject. Plots G–I represent the CMV-specific, and plots J–L the control culture-induced response in the seronegative subject. One of four representative experiments is shown.

from CMV seropositive donors. For these experiments, CMV or control Ag (BioWhittaker) activated whole blood samples were stained with CD3 PerCP (FL3 trigger),. CD8 FITC, and PE-conjugated anti-cytokine mAbs (anti-IL-2, anti-IFNγ and anti-TNFα). Fig. 2 shows representative three-color FACS plots comparing cytokine expression in CMV Ag (plots A–D) and control Ag (plots E–H) stimulated, CD3 gated T lymphocytes. In this example, the majority of the cytokine producing cells were observed within the CD8 − cell compartment, although significant IFNγ and TNFα expression was observed within the CD8 + cell population. Thus, 0.67% of the CD8 − and virtually none of the CD8 + T cells expressed IL-2, 2.60% of the CD8 − and 1.33% of the CD8 + T cells expressed IFNγ and 2.41% of the CD8 − and 1.86% of the CD8 + T cells expressed TNFα.

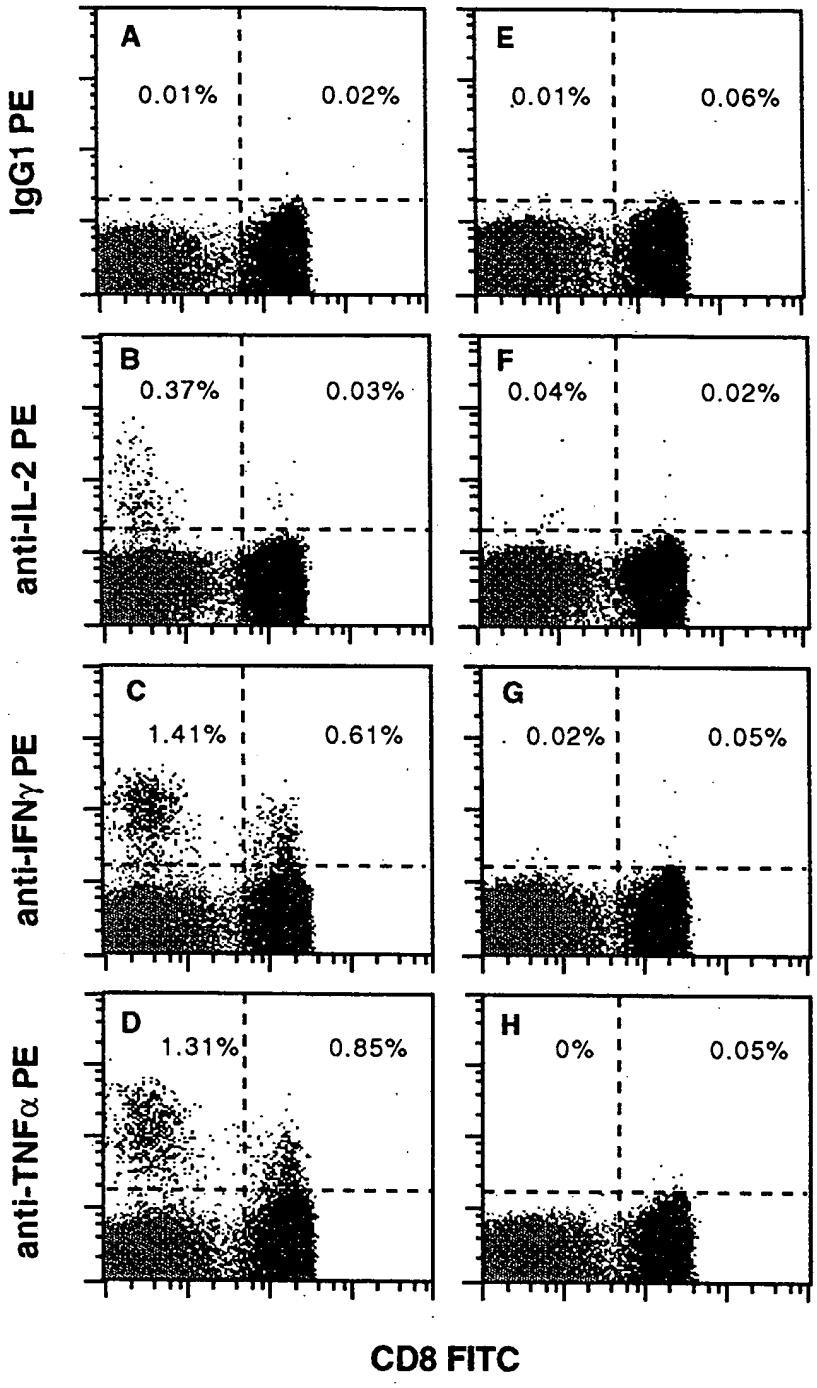

Fig. 2. CMV Ag-induced cytokine expression in CD8 + and CD8 − T cells (FL1 marker is set on the CD8 bright cell population). Whole blood from a CMV seropositive donor was stimulated with CMV viral or control Ag as described in Section 2. Blood samples were subsequently stained with CD3 PerCP, CD8 FITC and IgG1 PE isotype control, anti-IL-2, anti-IFNγ or anti-TNFα PE. A total of 50,000 CD3 + events were analysed for the upregulation of cytokines (blue and red dots). Plots A–D depict the CMV-specific response and plots E–H the control Ag-induced response. One of four representative experiments is shown.

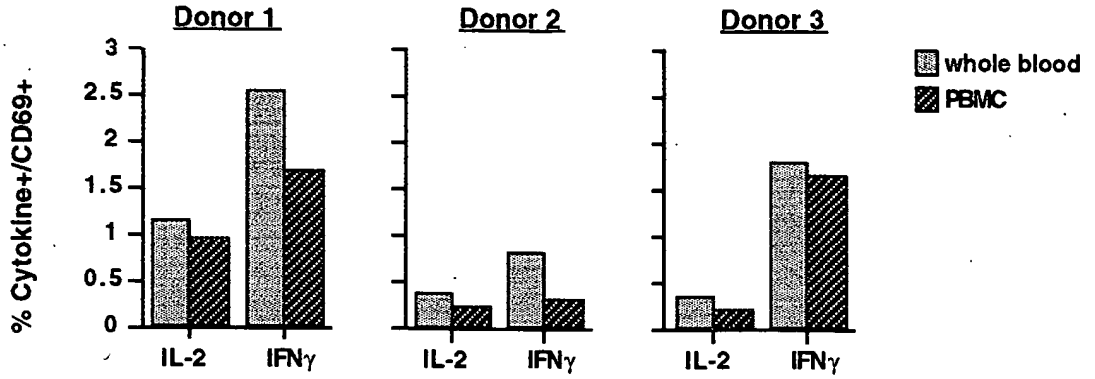

Fig. 3. Cytokine responses to CMV Ag measured in whole blood and PBMC cultures. Whole blood and PBMC in autologous plasma were stimulated with CMV Ag as described in Section 2. Activated samples were stained with CD4 PerCP, CD69 PE and anti-IL-2 or anti-IFNγ FITC. A total of 50,000 CD4 + T cells were analysed for the upregulation of CD69 and cytokines. Three of eight representative experiments are shown.

3.3. Cytokine responses to CMV Ag in whole blood and PBMC cultures

Detection of Ag-specific responses by flow cytometry was previously demonstrated in fractionated PBMC (Waldrop et al., 1997). To compare T cell responsiveness to specific Ag in whole blood and PBMC preparations, we examined the frequencies of cytokine producing cells responsive to CMV Ag under both culture conditions. Heparinized whole blood or PBMC in autologous plasma from CMV seropositive donors were stimulated with CMV viral Ag (BioWhittaker) for 6 h as described in Section 2. The PBMC culture tubes were incubated at a 5° slant from horizontal as previously described by Waldrop et al. (1997). Fig. 3 depicts IL-2 and IFNγ expression measured in either whole blood or PBMC cultures from three representative donors. The above donors were selected to illustrate the typical range of these cytokine responses. The cytokine profiles in whole blood and PBMC were generally comparable, however, higher frequencies of cytokine producing cells were consistently observed in whole blood cultures.

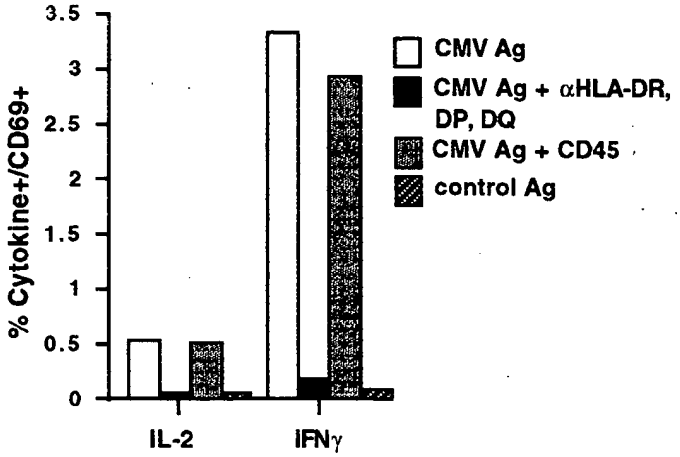

Fig. 4. Anti-class II MHC mAb-induced inhibition of Ag presentation and subsequent cytokine expression. Whole blood was stimulated with CMV Ag in the presence of anti-HLA-DR, -DP and -DQ. Activated cells were stained with CD4 PerCP, CD69 PE and anti-IL-2 or anti-IFNγ FITC. A total of 40,000 CD4 + T cells were analysed for the co-expression of CD69 and cytokines. One of three representative experiments is shown.

3.4. Inhibition of CMV Ag stimulation by anti-class II MHC mAbs

To demonstrate that these Ag-specific T cell responses were dependent on APC function, we tested the blocking of the class II MHC driven CD4 + cytokine expression in response to CMV Ag. A mixture containing three anti-class II MHC mAbs (anti-HLA-DR, anti-HLA-DP and anti-HLA-DQ; 10 $\mu g/10^6$ cells) was preincubated with whole blood for 15 min at RT. The whole blood cultures were subsequently stimulated with CMV or control Ag (BioWhittaker) for 6 h as explained in Section 2. Fig. 4 illustrates the level of inhibition exerted by the anti-class II mAbs in a single representative donor. The CD4 + IL-2 expression was inhibited by 87% and IFNγ expression by 95% in the cultures containing the anti-class II mAbs. A separate whole blood culture, stimulated in the presence of an antibody (CD45) that does not bind class II MHC determinants, showed no inhibitory effect on IL-2 expression and a negligible (11%) inhibitory effect on IFNγ synthesis. No inhibition of CD8 + cytokine production by the above anti-class II mAbs was observed (data not shown).

4. Discussion

Methods for analysis of T cell responses to specific antigen have traditionally relied on measurements of proliferation or cytokine expression in bulk cultures of PBMC in long term incubations with putative antigen (Clerici et al., 1993; ElGhazali et al., 1993; Croft et al., 1994; Cua et al., 1996). These techniques suffer from the drawback that they do not enable analysis of single cell responses in the context of unselected cellular backgrounds. In addition, these methods do not allow the assessment of the expression of more than one cytokine per cell, unless T cell clones are employed. Another limitation of cytokine measurements from supernatant fluids of bulk cultures is the inability to detect responses in very low frequency populations.

We have recently described a highly sensitive flow cytometric technique, that is based on the ability to detect single cell expression of cytokines, to simultaneously quantitate and phenotypically characterize antigen-specific memory/effector T cells in PBMC cultures (Waldrop et al., 1997). We also demonstrated, that using this approach, individual memory T cells expressing cytokines in response to specific antigen could be monitored at frequencies of less than 1%.

In a number of clinical applications, it is desirable to be able to measure the in vivo effects of drugs and immunomodulators on cellular immunity. These effects may influence T cell responses directly as a consequence of plasma concentration of drugs or in some situations, as a secondary effect of the cellular environment. It is therefore important to be able to measure antigen-specific responses at the single cell level in the context of changes in micro-environmental conditions.

In this report, we describe a simplified procedural modification of our original flow cytometric method which enables the rapid detection of low frequency Ag-specific memory T cells expressing cytokines in whole blood. As previously characterized with PBMC preparations (Waldrop et al., 1997), T cells responding to CMV antigen co-expressed CD69 and cytokines. The method outlined in this report allows the identification and characterization of T cells responding to either crude or purified preparations of CMV after short incubations in whole blood.

Similar to our previous observations with PBMC preparations, the whole blood response was shown to be antigen-specific, as determined by a number of critical parameters. Thus, cytokine expression was observed only in CMV-activated samples from CMV seropositive individuals, and was not observed in samples from seronegative individuals (Fig. 1), demonstrating host specificity for this response. The control antigen did not elicit significant T cell cytokine responses in any of the samples tested. Furthermore, we have recently demonstrated specific CD4 + cytokine responses to KLH in whole blood cultures from individuals immunized with KLH, but not in cultures from unimmunized subjects, also demonstrating host specificity as measured with a neoantigen (V. Maino, R. Levy, unpublished observations). Additionally, consistent with an Ag-specific response, CD4 + IL-2 and IFNγ expression detected with this assay was suppressed by antibodies to class II MHC products (Fig. 4), and to co-stimulatory/adhesion molecules (CD18, CD80) that are known to

participate in Ag-specific T cell responses (data not shown). Finally, in agreement with our previous observations with PBMC preparations (Waldrop et al., 1997), the responding CD4 + T cells were restricted to the $CD45RA^{low}/CD45RO^{high}$ memory cell compartment (data not shown).

A number of key observations minimize the possibility that bystander activation contributes to the observed frequencies of T cells responding to antigen. First, the presence of Brefeldin A in this short term assay prevents the expression of activation antigens and the secretion of factors (e.g., IL-12) which may contribute to non-specific activation of bystander T cells. Secondly, only T cells within the $CD45RO^{high}$ subset are activated by Ag. Finally, our previous studies with the superantigen SEB show that cytokine responses are restricted to T cells expressing the appropriate Vβ chain (Maino et al., 1995a; Waldrop et al., 1997). Although this response is not class II MHC-restricted, binding of SEB to both class II MHC antigen and specific TCR-Vβ is required for activation.

We report on antigen-specific responses to CMV in this manuscript because host specificity can easily be addressed as a control to validate the assay. As described in our previous report (Waldrop et al., 1997), frequencies of T cells responding to this chronic antigen are typically higher than responses to other recall antigens, such as mumps or tetanus. However, using this flow cytometric methodology, the frequencies to all antigens may be higher than earlier estimates of T cell repertoires, because this assay is more sensitive than earlier techniques, and it defines very early activation events (< 6 h) that precede the onset of apoptosis. In this regard, it is now well documented that most memory T cells respond to antigen with an effector response and then succumb to apoptosis prior to any possible proliferative response (Kabelitz et al., 1993; Russell, 1995). Furthermore, earlier estimates of antigen-specific T cell frequencies that are based on limiting dilution assays, may have underestimated total responding cells as compared to this short term assay, because of errors due to cell death and biased cell selection inherent in long term limiting dilution assays.

In addition to CMV, we have observed CD4 + cytokine responses to other viral antigens including mumps and measles, the bacterial antigen tetanus toxoid, and the purified protein antigen KLH. The frequencies of responding cells to these less ubiquitous Ags were significant, but usually 3–10-fold lower than responses observed with CMV. The frequencies of background events in the CD4 + T cell population (unstimulated, control Ag-stimulated or host negative control) were typically between 0% and 0.05%.

In agreement with previous studies (Linsley et al., 1991; Schwartz, 1992; Linsley and Ledbetter, 1993; Waldrop et al., 1997), we observed enhancement of the Ag-specific response by the co-stimulatory mAb CD28, and no direct stimulation of T cells by CD28 alone. The CD28-enhanced response was typically two-fold higher (% cytokine + /CD69 + cells) than the response to Ag in the absence of co-stimulus. Therefore, CD28 was included in all experiments in this study. The mechanism of the CD28-mediated increase in frequencies of T cells responsive to antigen appears to be due to differences in the co-receptor signalling requirements within subsets of T cells specific to processed CMV peptides (Waldrop et al., in preparation).

In addition to minimizing sample preparation time, the whole blood method described in this report introduces modifications that simplify the procedure. We added FACS™ Lysing Solution directly to activated whole blood cultures prior to permeabilization with FACS™ Permeabilizing Solution. FACS™ Lysing Solution not only causes red cell lysis but also promotes fixation of white cells, which is necessary for subsequent permeabilization and intracytoplasmic staining steps. Additional modifications include the culture of whole blood samples in upright conical polypropylene tubes, and the addition of 2 mM EDTA directly to blood cultures prior to the lysis/fixation step. We found that these steps were important to achieve reproducible recoveries of antigen-activated T cells following stimulation in whole blood. The culture of whole blood in polypropylene (instead of polystyrene) tubes yielded higher, more consistent recoveries of Ag-activated cells. This could be due to a more efficient detachment of activated cells, which tend to adhere to the sides of the culture tube, from polypropylene surfaces.

Although most significant cytokine expression was observed in CD4 + memory T cells following acti-

vation by antigen, we also observed significant, but lower frequencies of CD8 + T cells expressing IFNγ and TNFα. However, CD8 + T cells did not express IL-2 in response to CMV (Fig. 2). Although soluble antigen typically primes CD4 + T cells through the class II MHC dependent pathway, it has been reported by a number of investigators that CD8 + T cells can also respond to soluble antigens via class I MHC presentation pathways (Carbone and Bevan, 1990; Kovacsovics-Bankowski et al., 1993; Germain, 1994; Harding and Song, 1994; Reimann et al., 1994; Reise Sousa and Germain, 1995). The ability to identify CD8 + T cell responses at the single cell level using this methodology will enable further characterization of the T cell subsets involved in responses to exogenous antigen.

To investigate the effects of PBMC fractionation on the detection of antigen-specific responses, we compared the frequencies of CD4 + T cells responding to specific antigen in whole blood and PBMC in autologous plasma. This analysis suggested that although some variation was observed, similar results could be obtained with either assay format. Thus, donor responses that generated high frequencies of CMV-responding cells in PBMC preparations also demonstrated high frequencies when measured in whole blood cultures (Fig. 3). It should be noted, however, that we consistently observed slightly higher frequencies of responding T cells in whole blood cultures. In this comparison, 100% autologous plasma was employed as the source of supplemental serum, which contrasts with the method described in our previous report, in which RPMI-1640 and fetal bovine serum (FBS) were employed as the culture medium (Waldrop et al., 1997). Direct comparisons of T cell responses in whole blood using the methods described in this report and PBMC in FBS containing medium using the procedures described previously, also demonstrated consistently higher frequencies of antigen-specific T cells in whole blood cultures (data not shown).

The basis for higher observed frequencies of responding T cells in whole blood cultures is not clear. However, we have noted that over a 5–6-h culture period, whole blood cultures differentially sediment an upper band of PBMC over a red cell layer, possibly providing a more physiological environment in which to respond to stimuli. The sedimentation may also contribute to improved cell–cell contact within the PBMC population, unaffected by the presence of the lower layer of red blood cells. Preliminary results suggest that apoptosis is not significant within the 6-h culture period defined for this assay (B. Mehta, V. Maino, unpublished observations).

In summary, we have described a flow cytometric method which allows the identification and enumeration of T cells responding to specific antigen after a brief culture in whole blood. The modified technique described in this report is compatible with simple and rapid analysis of clinical samples and provides a means to directly examine the effects of in vivo drug concentrations on T cell immunity. Studies are in progress to examine the sensitivity of this cellular assay to drugs and other therapeutic modalities in clinical samples.

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(x) Related proceedings appendix

There are no related proceedings identified pursuant to 37 C.F.R. §41.37(c)(1)(ii).

(xi) Table of Authorities

Cases

(xii) Conclusion

In view of the arguments and authorities set forth herein, this Board should find the Final Rejections of the appealed claims to be in error and should reverse them.

Respectfully submitted,

3/20/2007
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